The Biochemistry of the Visual Cycle†

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I. Introduction: Overview of the Visual Cycle

Vertebrate vision begins with the absorption of light by rhodopsin, causing its 11-*cis*-retinal Schiff base chromophore to photoisomerize into its all-trans form. All-*trans*-retinal is liberated from rhodopsin in photoreceptor cells and is rapidly and enzymatically reduced to all-*trans*-retinol (vitamin A). For vision to continue, 11-*cis*-retinal must be regenerated in the eye, as this is the only organ in the body where 11-*cis*-retinoids are found. The actual biological site of isomerization is in the retinal pigment epithelium (RPE), the organ found behind the retina in vertebrates.

When vitamin A is produced in the photoreceptors, it is transported into the RPE where it is esterified by lecithin retinol acyl transferase (LRAT) and isomerized with hydrolysis to 11-*cis*-retinol. Oxidation by 11-*cis*-retinol dehydrogenase in the RPE to 11-*cis*-retinal completes the visual cycle. Molecular analysis of the various enzymes of the visual cycle has been hindered because they are membrane-bound and difficult to isolate. However, several of the enzymes have by now been isolated, cloned, and studied. In this review, novel aspects of the recent molecular enzymology of the visual cycle, particularly with respect to LRAT, are described.

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A. Processing of Vitamin A in the Retina

In all sighted species, vision begins with the photoisomerization of rhodopsin, a membrane-bound protein found in photoreceptors.[1−5] The chromophore of rhodopsin is an 11-*cis*-retinal Schiff base (Scheme 1). 11-*cis*-Retinal is a vitamin A derivative, whose only known function is in vision. The absorption of light by rhodopsin results in the cis to trans isomerization of its chromophore, causing a conformational change in rhodopsin. The resulting conformational change in photoactivated rhodopsin, spectroscopically defined as metarhodopsin II, leads to the activation of the phototransduction cascade and the initiation of the visual response (Scheme 1). After this process, the all-*trans*-retinal Schiff base is hydrolyzed, yielding the photochemically inactive protein opsin and all-*trans*-retinal. For opsin to function in vision, it must reunite with another 11-*cis*-retinal molecule to produce rhodopsin. The all-*trans*-retinal molecules liberated from photochemically activated rhodopsin molecules must be directly or indirectly enzymatically isomerized to 11-*cis*-retinal for vision to proceed. The

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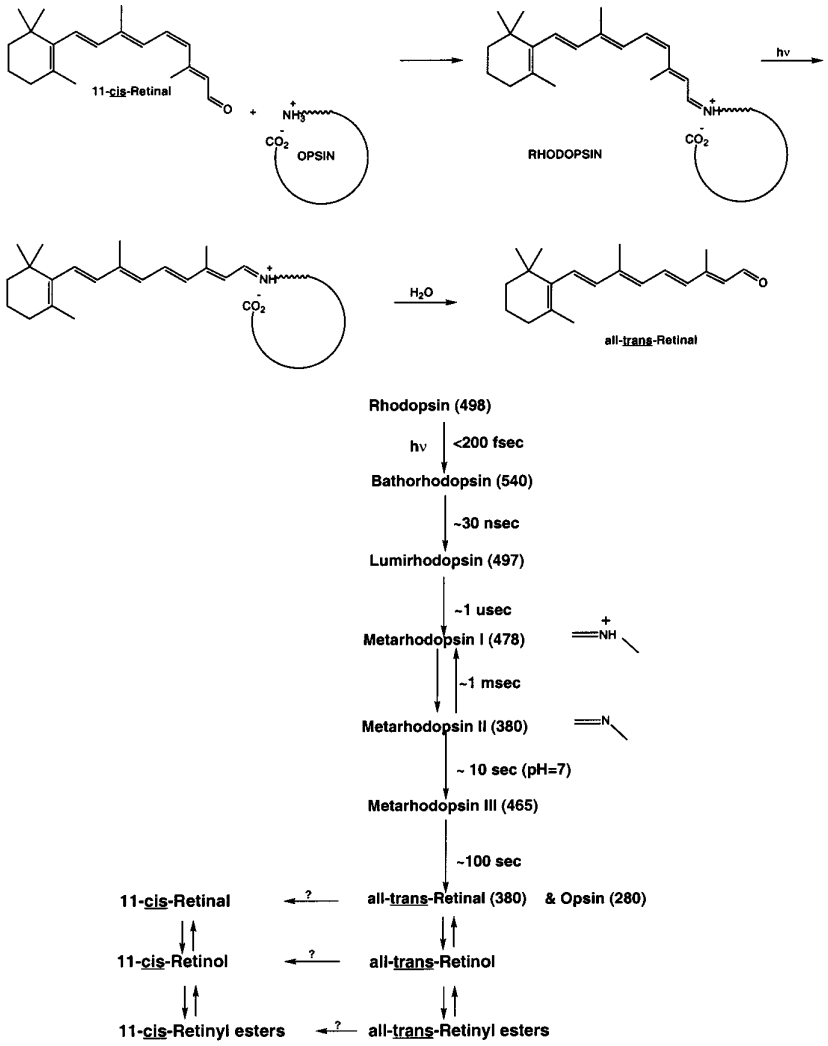
Scheme 1. The Photoisomerization of Rhodopsin and the Biosynthesis of Its Chromophore 11-*cis*-Retinal



visual cycle comprises the series of enzymatic reactions linking all-*trans*-retinal to the chromophore of rhodopsin 11-*cis*-retinal (Scheme 1).

The need for this isomerization pathway occurs in vertebrates because the photoisomerization of the protonated Schiff base of 11-*cis*-retinal to its all-trans congener is irreversible (Scheme 1). As indicated above, after photoisomerization of the 11-*cis*-retinal Schiff base, the all-*trans*-retinal Schiff base of rhodopsin hydrolyzes, generating all-*trans*-retinal. The all-*trans*-retinal is short-lived in photoreceptors, partly because it is chemically highly reactive and can form undesired Schiff base derivatives along with products derived from Schiff base formation.[6] The liberated all-*trans*-retinal is rapidly reduced in photoreceptors. Enzyme catalyzed reduction of the all-*trans*-retinal by a recently identified member of the long chain alcohol dehydrogenase family,[6,7] results in the formation of all-*trans*-retinol (vitamin A). All of the substrate processing thus far described occurs in the photoreceptors proper (since most of the vision biochemistry described thus far relates to rod dominated species, the results described in this review will of necessity relate to rod dominated vision and the biochemistry that obtains in rod cells). Thus, after the generation of vitamin A in rod cells, the vitamin A leaves the rod cells and is transported to the retinal pigment epithelium (Scheme 2).

B. Reisomerization of Vitamin A in the RPE

The retinal pigment epithelium looms large in the processing of retinoids in vision. It has been known for years that the retina itself shows little or no capacity for 11-*cis*-retinal regeneration after the bleaching of rhodopsin.[1,4,8,9] Photochemical bleaching of rhodopsin, of course, involves the photochemical isomerization of the 11-*cis*-retinal Schiff base followed by its hydrolysis to yield the protein opsin and all-*trans*-retinal. The only defined retinoid processing activity thus far found in the retina is the all-*trans*-retinol dehydrogenase alluded to above.[6,7,10−12] All of the further enzymatic processing of retinoids occurs in the retinal pigment epithelium (RPE) as illustrated in Scheme 2. This point was clearly demonstrated when it was shown that extracts from bovine RPE, but not retina, had the capacity to process exogeneously added vitamin A into 11-*cis*-retinoids.[13]

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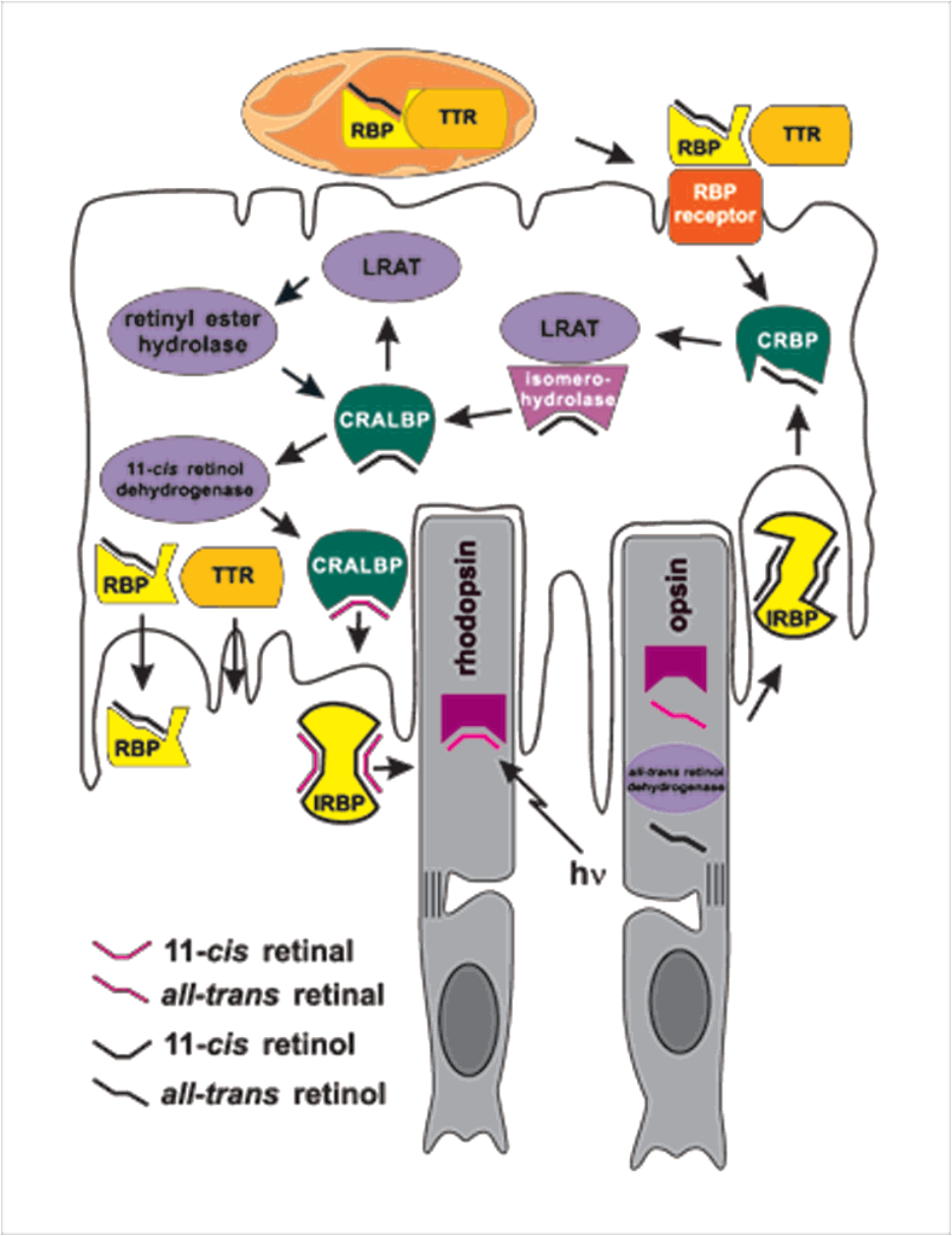
Scheme 2. Fluxes of Vitamin A in the Visual Cycle and the Role of the Retinal Pigment Epithelium in the Biosynthesis of 11-*cis*-Retinoids[a]



[a] The above scheme illustrates the processing of retinoids in the retina/retinal pigment epithelium. Several retinoid binding proteins are indicated including RBP (retinol binding protein), CRALBP (cellular retinaldehyde binding protein), CRBP (cellular retinol binding protein), TTR (transthyretin), and IRBP (interphotoreceptor retinol binding protein).

This was the first demonstration of an in vitro system capable of processing vitamin A into an 11-*cis*-retinoid.[13]

The vitamin A generated in the photoreceptors must then be transported into the RPE for further processing into 11-*cis*-retinoids. A major protein, called IRBP, found in the interphotoreceptor space, is thought to play a major role in this transport.[14−18] This protein, which binds many amphipathic substances with similar affinities, may be an important part of the retinoid shuttling pathway. In vitro eye cup experiments certainly suggest an important role for this protein.[19] However, IRBP knockout mice do not show a profound visual phenotype, suggesting that either IRBP is not an essential transporter or that other proteins compensate for it in its absence.[20,21] It should also be noted though that vitamin A, while it is certainly a hydrophobic alcohol, undergoes intermembranous transfer at appreciably high rates.[22,23] Thus, a mobilizing protein for vitamin A is not essential on kinetic grounds alone. There are other reasons why a binding protein might be involved, however. Directionality is one. It might be important to ensure that the liberated vitamin A is transported to where it is required for further enzymatic processing. In addition, vitamin A is readily

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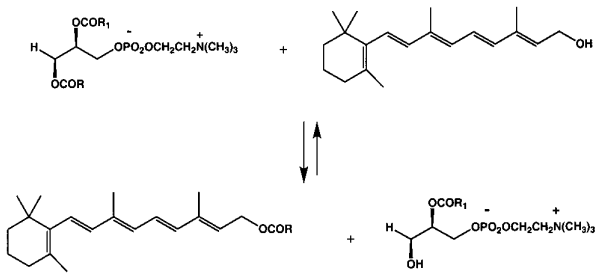
Scheme 3. Transesterification of Vitamin A Catalyzed by Lecithin Retinol Acyl Transferase (LRAT)

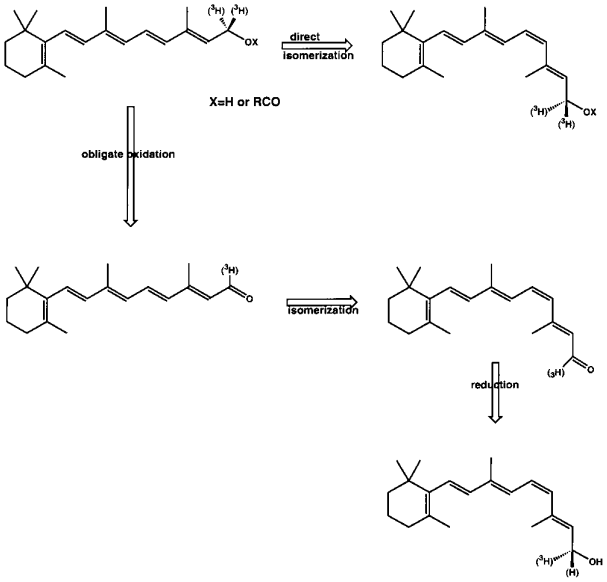


degraded by oxygen-dependent mechanisms in aqueous milieu.[24] A binding protein could prevent the oxidative destruction of vitamin A in a buffered medium. At this point, however, it is impossible to be certain about the role of IRBP in retinoid transport.

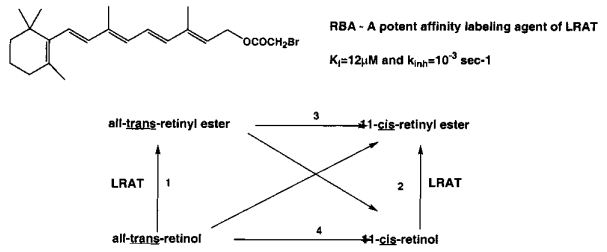
Once the vitamin A makes its way from the retina to the RPE, it needs to enter the RPE cells for further processing. There are differing notions on how this might occur. There is the clear possibility of passive diffusion across the RPE membranes and the alternative possibility of a receptor-mediated event.[25,26] A receptor-mediated event might involve an IRBP receptor.[25,26] While this possibility has been discussed in the literature, little if any evidence has been advanced that would support this notion. At this point, the most parsimonious explanation for vitamin A entrance into the RPE occurs through a passive process.

Once the vitamin A penetrates RPE cells, it is esterified by an enzyme called lecithin retinol acyltransferase (LRAT) (Scheme 3).[27−29] This novel enzyme, which is central to the visual cycle, catalyzes a transesterification reaction in which an acyl group from the *sn*-1 position of phosphatidylcholine (lecithin) is transferred to vitamin A to generate retinyl esters.[27−29] The overall reaction for this pathway is shown in Scheme 3. For each all-*trans*-retinoid found in the RPE there is a corresponding 11-*cis*-retinoid (Scheme 1). This complicates analysis of the actual isomerization pathway since there are nine possible routes given the three possible substrates and the three possible products. However, double-labeling experiments considerably simplified analysis of the pathway to 11-*cis*-retinoids.[30] These experiments unequivocally showed that isomerization occurs at the alcohol level of oxidation.[30] That is, the isomerization reaction must involve either vitamin A itself, all-*trans*-retinyl esters, or a thus far unidentified retinol derivative as the isomerization substrate.[30] This fact was established by double labeling experiments using 15-^{3}H, 15-^{14}C-vitamin A as a precursor molecule for isomerization and demonstrating that isomerization occurs in the absence of ^{3}H loss.[30] Had all-*trans*-retinal been the substrate, approximately 50% of the ^{3}H would have been lost in the initially formed 11-*cis*-retinoids.[30] A diagram summarizing the experimental approach leading to the conclusion that isomerization must occur at the alcohol oxidation state is shown in Scheme 4. Moreover, the use of inactivators of LRAT also demonstrated that the initially formed 11-*cis*-retinoid is 11-*cis*-retinol.[31]

Scheme 4. Tritium Release Experiments Show that Isomerization Must Occur at the Alcohol Oxidation State



Scheme 5. The LRAT Affinity Labeling Agent, all-*trans*-Retinyl-α-bromoacetate (RBA) and Its Role in Elucidating Substrate and Product in the Isomerization Process[a]



[a] RBA blocks steps 1 and 2. When all-*trans*-retinyl esters are first allowed to form followed by the addition of RBA, 11-*cis*-retinol is produced, eliminating step 3 from consideration. Preincubation with RBA followed by the addition of all-*trans*-retinol results in no isomerization, eliminating step 4 from consideration.

Inhibition of LRAT blocks the formation of 11-*cis*-retinyl esters and reveals the initial formation of 11-*cis*-retinol in the absence of its further esterification.[31] With these kinds of experiments, one is only left with three possible isomerization pathways given the known retinoid entities in the RPE: between retinyl esters, between retinols, or a pathway from all-*trans*-retinyl esters to 11-*cis*-retinol. Experiments using LRAT inactivators show that in several species, retinyl esters play a central role in the isomerization pathway. The evidence implying a central role for esters in the isomerization pathway is provided below.

Vitamin A esterification has been classically considered essentially a storage reaction in the visual cycle. However, esterification appears to play a much more important role here. Inhibition of LRAT activity by the affinity labeling agent all-*trans*-retinyl bromoacetate (RBA) (Scheme 5) leads to the inability of RPE membranes to process added vitamin A into 11-*cis*-retinoids in bovine RPE membranes.[31] However,

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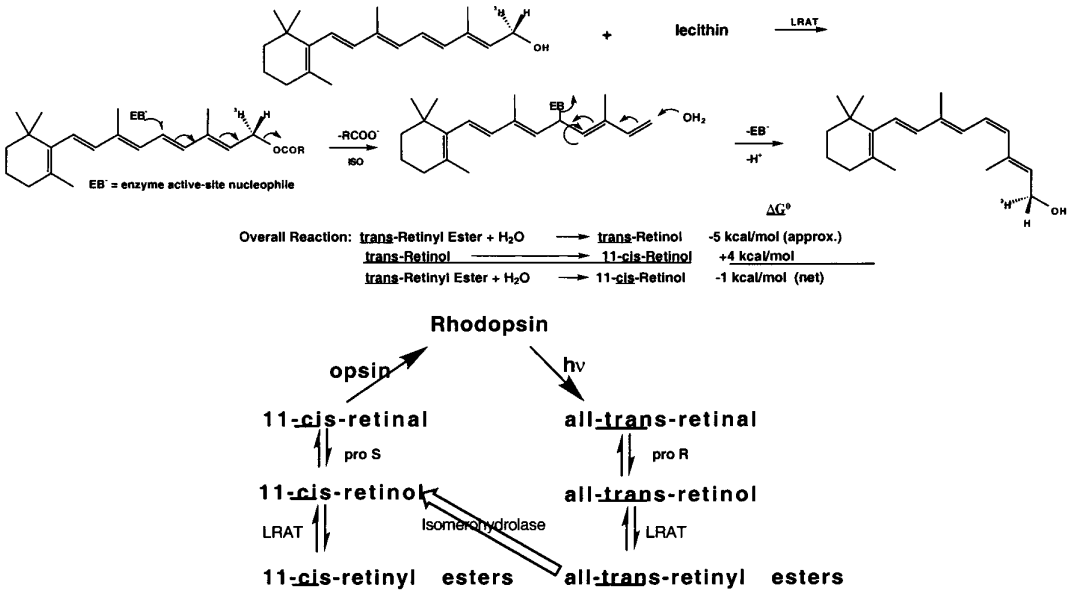
Scheme 6. The Energy that Drives the Uphill Isomerization of all-*trans*-Retinoids Comes from the Hydrolysis of Retinyl Esters[a]



[a] In the diagram shown here, a coupled reaction between ester formation and isomerization provides the energy to drive the biosynthesis of 11-*cis*-retinol. The lower section of the scheme shows the visual cycle and the proposed reaction pathway to 11-*cis*-retinol.

if all-*trans*-retinyl esters are generated from vitamin A before the addition of RBA, then isomerization proceeds normally, and 11-*cis*-retinol is generated as the product.[31] This means that RBA, which inactivates LRAT, inhibits isomerization as a consequence of this inhibition, rather than having a direct effect on isomerization per se. RBA has only a very weak effect on isomerization activity.[31] These kinds of experiments have been repeated on mouse RPE and on amphibian RPE with the same results. Interestingly, a recent report also demonstrates the same results in the crayfish *Procambarus clarkii*, again showing the obligate importance of retinyl ester formation for isomerization and demonstrating a convergence of overall isomerization mechanism in distantly related organisms.[32]

The experiments described above immediately require an explanation as to why retinyl esters might be essential for isomerization. This issue goes back to the first recognition that an energy source is required for the formation of 11-*cis*-retinoids.[33] At chemical equilibrium, 11-*cis*-retinoids only comprise 0.1% of an equilibrium mixture.[34] This means there is a 4 kcal/mol difference between 11-*cis*-retinoids and their all-trans congeners.[34] Since dark adapted animals possess predominantly 11-*cis*-retinoids, it is clear that the in vivo situation resides very far from equilibrium.[35] Therefore, an energy source must be posited to drive this process.

The simplest mechanism by which retinyl esters could be involved in the energy transduction step is through the coupling of the free energy of hydrolysis of retinyl esters to the thermodynamically uphill isomerization process.[33] Acyl esters have free energy of hydrolyses in the −5 kcal/mol range,[36] and the difference in free energy between all-*trans* and 11-*cis*-retinoids is approximately −4 kcal/mol.[34] The simplest mechanism for coupling the energies involves an isomerohydrolase enzyme, which directly processes all-*trans*-retinyl esters into 11-*cis*-retinol (Scheme 6).[33,37,38] That the isomerization reaction must be enzyme mediated comes from stereochemical studies where it was shown that isomerization proceeeds with inversion of stereochemistry at C15.[33,37] A chemical process would not alter stereochemistry at C15. Inversion of stereochemistry at C15 is also consistent with the mechanism shown in Scheme 6.

Studies with the LRAT inactivator RBA clearly demonstrate that 11-*cis*-retinol is the first product formed as a result of isomerization.[31] While a concerted isomerization/hydrolysis reaction represents the simplest energy coupling mechanism, it is by no means the only possible mechanism. For example, hydrolysis of the 11-*cis*-retinyl ester could occur after isomerization via a specific 11-*cis*-retinyl ester hydrolase with the same thermodynamic consequences. Interestingly, some evidence for enzymes of this type have been put forward in the literature.[39,40]

Other possible isomerization scenarios include mechanisms in which all-*trans*-retinyl esters are intermediates along pathways that ultimately require a transesterification type process to drive the isomerization reaction. The demonstration that isomerization occurs at the alcohol level of oxidation is of interest here because obvious mechanisms of energy transduction are available at the alcohol level of oxidation which are unavailable with all-*trans*-retinaldehyde. For example, phosphorylation or acylation of the alcohol moiety gives rise to potentially high-energy intermediates that could provide the energy to drive thermodynamically uphill isomerization events. With respect to phosphorylation, no evidence for retinyl phosphate has ever been described in the RPE even though it has been searched for.[13,41] Moreover, neither ATP, GTP, nor other high-energy phosphate containing molecules enhance 11-

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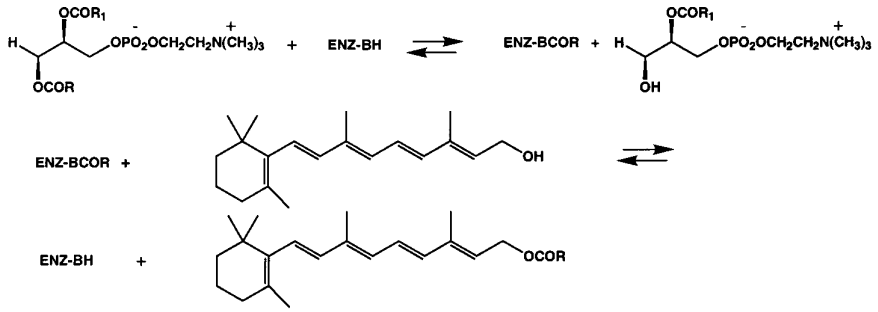
Scheme 7. LRAT Operates by a Ping-Pong Kinetic Mechanism



cis-retinoid formation using RPE membranes.[13,42] Thus, if all-*trans*-retinyl phosphate is involved as an intermediate in 11-*cis*-retinoid biosynthesis, its involvement is not readily verified experimentally. As the isomerizing activity has thus far not been identified, all of these possibilities need to be considered, although the most parsimonious mechanism given the data available involves retinyl esters as an important substrate in the isomerization pathway. In conclusion, among the data that supports the hypothesis that retinyl esters are obligate intermediates in the overall synthesis of 11-*cis*-retinol are (i) retinoid double-labeling experiments demonstrate that isomerization must occur at the alcohol level of oxidation i.e., retinol (ester) stage and eliminates an isomerization event occurring between all-*trans* and 11-*cis*-retinal; (ii) the use of structurally distinct LRAT inactivators establishes that 11-*cis*-retinol is the product of the isomerization event; and (iii) the use of the previously mentioned LRAT inactivators shows that isomerization in RPE membranes (i.e., 11-*cis*-retinol formation) is blocked from added all-*trans*-retinol, but not from preformed all-*trans*-retinyl esters.

Although the enzyme(s) catalyzing the isomerization reaction has not been identified thus far, considerable advances in the biochemistry of the visual cycle have been made in the last several years. In the next sections, the molecular enzymology of some of the important enzymes already cloned and studied will be discussed. One of the daunting issues confronting studies on the visual cycle is the fact that most of the enzymes involved are membrane bound and are minor constituents. While there have been great advances made in the techniques available to biochemistry over the past 10 or so years, these advances have unfortunately not included methodologies for the purification of minor membrane constituents. A major point in this review is that there may be substantial surprises in the study of these proteins. This point is readily exemplified in molecular studies on lecithin retinol acyl transferase.

C. Lecithin Retinol Acyl Transferase (LRAT)

LRAT is an essential, membrane-bound enzyme in the visual cycle and provides the substrate for isomerization, as well as generating a chemically stable storage form of vitamin A in the RPE. LRAT is also found in other organs of the body and has been strongly implicated in vitamin A uptake and delivery in general.[43] Thus, substantial quantities of LRAT are found in the intestine, liver, and other organs known to be active in vitamin A processing.[44] LRAT catalyzes a transesterification reaction that occurs between phosphatidylcholine (lecithin) molecules and vitamin A (Scheme 3).[27−29] The reaction is regiospecific, with transfer only occurring from the *sn*-1 position of lecithin.[45] Also lecithin is by far the most active of the common phospholipids with respect to acyl donation.[45] While there is striking discrimination found with respect to phospholipid headgroup type and the position of the acyl moiety to be transferred, there is little discrimination found with respect to the structure of the acyl moiety itself.[28] The fact that the biologically relevant retinyl esters found are largely of long chain fatty acids (steroyl and palmitoyl) simply reflects the fact that these are the fatty acids found at the *sn*-1 position of lecithin.[28] As mentioned above, the purification of LRAT has been vexing. Even without purifying LRAT, however, a substantial amount of biochemical and chemical information has accumulated on the protein. It is always important to establish a kinetic mechanism for an enzyme because this often provides insights into the underlying chemical mechanism of action of the enzyme. In the case of LRAT, it has been established that the enzyme operates by an ordered ping-pong mechanism in which the lecithin binds first and transfers an acyl group to the enzyme, followed by the departure of the lysophospholipid.[45] Vitamin A is then bound to the enzyme, accepts the acyl moiety, and departs as the retinyl ester.[45] The overall kinetic mechanism is depicted in Scheme 7.

This kind of mechanism requires an active-site nucleophile to accept and transfer the acyl moieties. In fact, acyltransferase enzymes usually operate by a serine or cysteine protease-like mechanism.[46] In this class of enzymes, a catalytic triad containing the active-site nucleophilic residue and the suitably placed aspartate and histidine residues are found.[47,48] The fact that acyltransferases and serine/cysteine proteases share common mechanistic features is understandable, because mechanistic convergence is expected in situations such as these. Here, the catalytic mechanisms would be expected to be similar, the major difference being that in the case of a protease, the attacking nucleophile is water, and in the case of a transferase the nucleophile will depend on the exact chemical reaction involved. For example, lecithin cholesterol acyl transferase (LCAT) is an enzyme that transfers an acyl group from the *sn*-2

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position of phospholipids to cholesterol to produce cholesterol esters.[46,49−51] This soluble enzyme is of substantial importance in the uptake and delivery of cholesterol and has been studied in some detail.[52] The enzyme has a typical serine protease catalytic triad motif as determined by site-specific mutagenesis.[53] The catalytic triad of LCAT involves Ser181, Asp345, and His377, and the enzyme contains the serine lipase motif of LI/VGHS.[53] LCAT is presumed to operate by this standard mechanistic paradigm expected of serine proteases/lipases.[53]

The overall similarity in the chemical reactions catalyzed by LCAT and LRAT would suggest that they operate by similar mechanisms. This turns out not to be the case. For one thing, while LCAT is readily inhibitable by serine directed, group-specific chemical reagents, LRAT is not.[28] In fact, the chemical inhibition profile for LRAT suggests that it is a thiol-dependent enzyme rather than a serine-dependent enzyme.[29] For example, LRAT is readily inactivated by organomercurials in the micromolar range.[28] Moreover, the sensitivity of LRAT to irreversible inactivation by the affinity labeling agent RBA also suggests the possibility of a cysteine residue essential for catalysis. The identification of the active-site nucleophile of LRAT depended, of course, on purification and cloning. To do this is not a straightforward exercise because LRAT is a minor membrane-bound protein. There are no generally useful approaches for the purification of proteins of this type, and new strategies had to be developed to obtain some direct sequence information on LRAT prior to its cloning and ultimately its full sequencing.

II. Molecular Enzymology of Visual Cycle Enzymes

A. Identification and Cloning of LRAT

LRAT, like other integral membrane-bound proteins, presents immense barriers to their purification. This is especially true for minor membrane components which, like LRAT, are found in the amounts of less than 1%. Identification and purification of membrane-bound proteins still presents a formidable problem in protein biochemistry. This is especially true if many integral membrane proteins operate by different mechanistic strategies than do their soluble counterparts. It should also be noted that it has been estimated that between 20 and 30% of the total proteins are likely to be membrane proteins.[54]

There are several issues to confront in purifying minor membrane constituents. Minor and major membrane components are distinguished for obvious reasons. For example, the integral membrane protein rhodopsin is approximately 75% of the total membrane proteins in retinal photoreceptor membranes.[5,55] The purification of this protein only requires the ability to solubilize the protein in detergent and carry out a simple column purification step. The situation with a minor membrane component, such as LRAT, estimated to be in the neighborhood of 0.1% of total RPE membrane proteins, presents much greater difficulties. While LRAT can be readily solubilized in a variety of detergents in a stable form, column chromatography invariably leads to progressive and irreversible denaturation of the enzyme.[28,56] This, unfortunately, is not an atypical scenario when working with membrane-bound enzymes. Whatever essential lipid−protein interactions are present in the membrane are lost upon solubilization and purification. While effective detergents make up for part of these interactions, they are often unable to completely supplant endogenous lipid−protein stabilizing interactions. Thus, as purification proceeds in time, the protein becomes increasingly unstable. Sometimes a water-soluble affinity ligand can be used to affect a substantial purification in a single step. Unfortunately, in the case of LRAT the substrates are hydrophobic, making the design of a hydrophobic inhibitor remote. Hydrophobic affinity ligands are not of much use for implementing substantial protein purification because they are too nonspecific in their interactions with proteins. The scenarios enumerated here, so typical for many membrane-bound proteins, were exactly found in the case of LRAT, rendering hopes at its full purification remote by standard techniques. A different strategy for the identification of LRAT was required, and this strategy involved its specific affinity labeling.

B. Biotin Affinity Labeling as an Approach to Identifying LRAT and the Molecular Basis of LRAT Action

LRAT could be effectively solubilized in detergents such as reduced Triton X-100 and purified routinely by factors of at least 25-fold.[28] A preparation of this type is very far from homogeneity as judged by SDS−PAGE. Moreover, the partially purified protein is quite unstable, preventing its further purification. Consequently, new methods were required to identify and clone LRAT.

As mentioned above, LRAT is effectively affinity labeled by all-*trans*-retinyl-α-bromoacetate (RBA) (Scheme 5) in the low micromolar range.[31] In fact, using ^{3}H-RBA a major band at approximately 25 kDa was labeled.[45] However, the harvesting of the labeled protein did not provide useful sequence information, as mixtures of proteins were found comigrating at approximately 25 kDa with LRAT after SDS gel electrophoresis. A further purification step was required. Earlier we had shown that biotin-containing affinity labeling agents could be used to specifically identify certain classes of enzymes.[57] As applied to LRAT identification, the analogue *N*-boc-L-biocytinyl-11-aminoundecane (BACMK) shown in Scheme 8 labeled LRAT in the low micromolar range.[57,58] The adducted biotin moiety allowed for two advantages over standard affinity labeling agents which are both based on the enormous (approximately 10^{15} M^{-1}) affinity constant of the protein avidin for biotin.[57,58] This high affinity facilitates the purification of the labeled protein using avidin affinity chromatography, and, second, the biotin moiety allows for the facile detection of the labeled protein on gels using avidin conjugated to an enzyme capable of generating chemiluminiscent products. A flow diagram describing this procedure in functional proteomics is also

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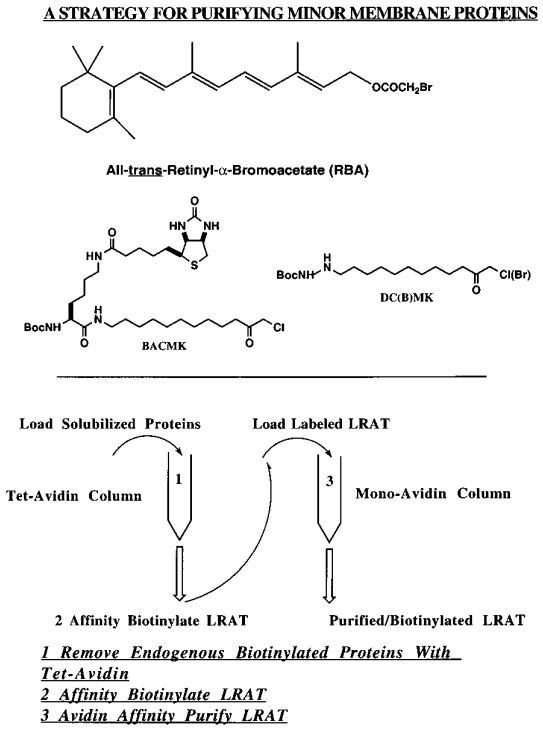
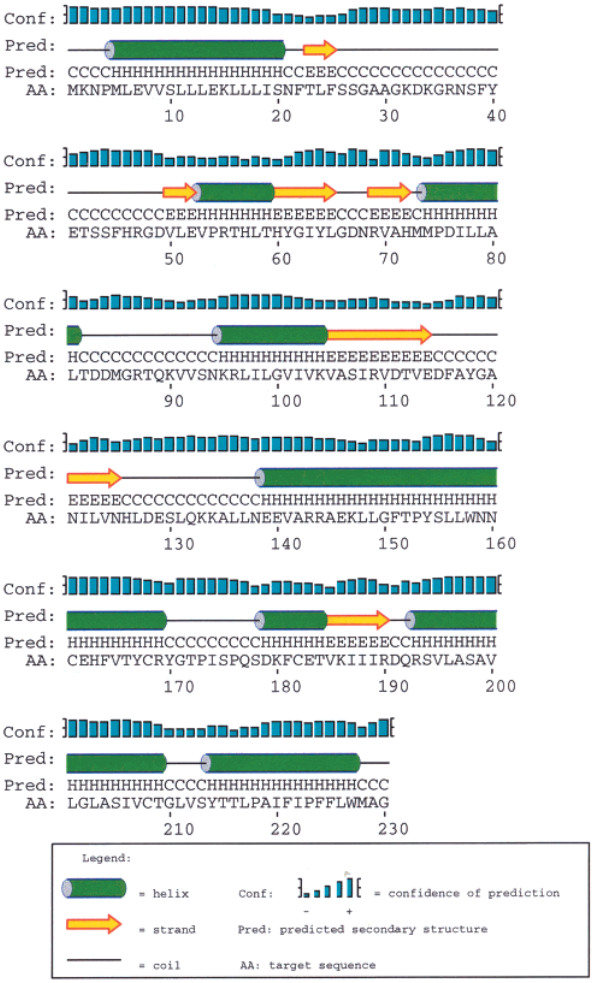
Scheme 8. The Affinity Biotinylation of Lecithin Retinol Acyl Transferase[a]



[a] Solubilized RPE proteins are first put through a tetrameric avidin column to remove all endogenously biotinylated proteins after labeling LRAT with BACMK (BACMK refers to *N*-Boc-L-biocytinyl-11-aminoundecane chloromethyl ketone), the biotinylated LRAT is purified by avidin affinity chromatography.

shown in Scheme 8, using the affinity-labeling agent BACMK. This analogue is approximately as active as RBA in inactivating LRAT. In the overall process, solubilized RPE membranes are first put through an avidin affinity column to remove all endogenously biotinylated proteins.[58] LRAT is not bound to this kind of column. In subsequent steps, the solubilized LRAT is labeled with BACMK, and then purified on a monomeric avidin column, using biotin as the eluant.[58] When the eluted proteins are assessed by SDS−PAGE electrophoresis, a single protein at approximately 25 kDa was observed. Microsequencing led to the identification of two novel peptide fragments (the N-terminal sequence of MKNPMLEAVSLVLEKLLFISYKKF and an internal sequence of HLTHYGIYLGDNR) from LRAT not found in any known protein at the time.[58] Having the peptide sequences made it possible to clone and sequence the human cDNA for LRAT, providing the sequence of an entirely novel protein (Scheme 9).[58] The predicted secondary structure of LRAT is also provided in this scheme. Interestingly, no homology at all was found to LCAT, the soluble, serine protease-like enzyme capable of acylating cholesterol with lecithin. When the cDNA was transfected into HEK-293 cells, the membranes of these cells contained robust LRAT activity found to be absent in the HEK-293 cells themselves and in HEK-293 cells transfected with empty vector.[58] These experiments prove that the cDNA of putative LRAT is indeed of native LRAT.[58]

Scheme 9. The Sequence of Human LRAT along with Its Predicted Secondary Structure[a]



[a] C refers to coil, H refers to helix, and E refers to extended.

LRAT is composed of a polypeptide of 230 amino acids with a calculated mass of 25.3 kDa with a novel sequence.[58] Interestingly, shortly after the protein was cloned it also appeared in the human genome data bank. Of course, in the latter case no function to the protein could be attributed inasmuch as it does not belong to a known family of enzymes or proteins. This may often be the case with minor membrane proteins that may not operate by mechanistic paradigms currently known. Therefore, genomics in the absence of functional proteomics may not be particularly informative.

Several interesting predictions can be made concerning the structure of LRAT given its sequence. First of all, hydropathy analysis clearly shows that LRAT has at least two transmembrane segments, at its N- and C-termini (Scheme 10). Some algorithms suggest the possibility of four transmembrane helices.[58] In any case, it is very clear that LRAT possesses structural features consistent with its being an integral membrane protein. By the usual biochemical criteria, LRAT behaves as an integral membrane protein. For example, LRAT sediments with the membrane fraction and is not extracted and rendered

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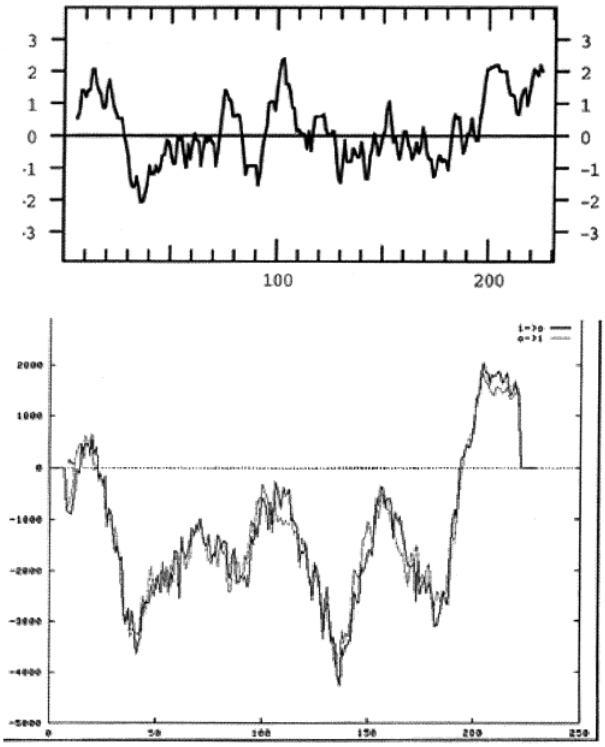
Scheme 10. Hydropathy Plots (upper-Kyte-Doolittle; lower TMPRED) for LRAT Showing Predicted Transmembrane Segments



Scheme 11. Proteins Showing Homologies to LRAT (TR10952371095237)

```
                  1       10        20        30        40
                  |--------+---------+---------+---------|
trl0952371095237                SSFHRGDVLEVPRTHLTHYGIYLGDNRV
Hs                MALARPRPRLGDLIEISRFGYAHWAIYVGDGYV
revrat              MPIPEPKPGDLIEIFRPMYSHWAIYVGDGYV
Hsrev             MRAPIPEPKPGDLIEIFRPFYRHWAIYVGDGYV
inducible         MASPHQEPKPGDLIEIFRLGYEHWALYIGDGYV
retinoic          MASPHQEPKPGDLIEIFRLGYEHWALYIGDGYV
mouse          MAVNDCFSLTYPHNPHPGDLIEVFRPCYQHWALYLGDGYV
Consensus         m......PhpGDLIE!fR..Y.HWAiYIGDGYV

                  41      50        60        70        80
                  |--------+---------+---------+---------|
trl0952371095237 AHMMPDILLALTDDMGRTQKVVSNKRLILGVIVKVASIRV
Hs               VHLAPA-----------SEIAGAGAASVLSALTNKAIVKK
revrat           IHLAPP-----------SEIPGAGAASIMSALTDKAIVKK
Hsrev            VHLAPP-----------SEVAGAGAASVMSALTDKAIVKK
inducible        IHLAPP-----------SEYPGAGSSSVFSVLSNSAEVKR
retinoic         IHLAPP-----------SEYPGAGSSSVFSVLSNSAEVKR
mouse            INIAPI-----------DGIRSS-FTSAKSVFSTKALVKM
Consensus        !H$AP.             s#i.gsg..S!lSVls.kA.VK.

                  81      90        100       110       120
                  |--------+---------+---------+---------|
trl0952371095237 DTVEDFAYGANILVNHLDESLQKKALLNEEVARRAEKLLG
Hs               ELLSVVAGGDNYRVNNKHDDRYTPLPSNKIVKRAEELVGQ
revrat           ELLRDVAGKDKYQVNNKHDKEYTPLPLNKIIQRAEELVGQ
Hsrev            ELLYDVAGSDKYQVNNKHDDKYSPLPCTKIIQRAEELVGQ
inducible        ERLEDVVGGCCYRVNNSLDHEYQPRPVEVIISSAKEMVGQ
retinoic         GRLEDVVGGCCYRVNNSLDHEYQPRPVEVIISSAKEMVGQ
mouse            QLLKDVVGNDTYRINNKYDTTYPPLPVEEVIQRSEFPIGQ
Consensus        #lLeDVaGGdnYrVNNk.D..Y.PlPv#e!!.RaeE.!GQ

                  121     130       140       150       160
                  |--------+---------+---------+---------|
trl0952371095237 FTPYSLLWNNCEHFVTYCRYGTPISPQSDKFCETVKIIIR
Hs               ELPYSLTSDNCEHFVNHLRYGVSRSDQVTGAVTTVGVA--
revrat           EVLYRLTSENCEHFVNELRYGVPRSDQVRDTVKVATVT--
Hsrev            EVLYKLTSENCEHFVNELRYGVARSDQVRDVIIAASVA--
inducible        KMKYSIVSRNCEHFVTQLRYGKSRCKQVEKAKVEVGVA--
retinoic         KMKYSIVSRNCEHFVAQLRYGKSRCKQVEKAKVEVGVA--
mouse            EVAYDLLVNNCEHFVTLLRYGEGVSEQANRAIGTIGLV--
Consensus        e.pYSLls#NCEHFVt.LRYG..rS.Qv#ka..TVg!a

                  161     170       180       190       200
                  |--------+---------+---------+---------|
trl0952371095237 DQRSVLASAVLGLASIVCTGLVSYTTLPAIFIPFFLWMAG
Hs               -AGLLAAASLVGILLARSKRERQ
revrat           -GVGLAALGLIGVMLSRNKKQKQ
Hsrev            -GMGLAAMSLIGVMFSRNKRQKQ
inducible        -TA-LGILVVAGCSFAIRRYQKKATA
retinoic         -TA-LGILVVAGCSFAIRRYQKKATA
mouse            -AAGIDIFTFLGLFPKRQRTKY
Consensus         .a.l.a..vlGl...r.r.......
```

soluble by a variety of chaotropic agents.[28] A secondary structure predictor of the LRAT structure is also consistent with the idea that the N- and C-termini of LRAT may be membrane associated. Both the N- and C-termini are predicted to be α-helical (Scheme 9). There are several other regions predicted to be α-helical, including the region between positions 138 and 170. This region contains a highly conserved C161 thought to be important for catalysis.[58]

As mentioned previously, LRAT possesses a unique sequence, which is surprising in light of the fact that the reaction it carries out is not so unusual. As mentioned above, LCAT carries out a very similar reaction but shows no homology to LRAT. A literature search on LRAT provides a grouping of several homologous proteins of unknown function.[58] Perhaps the most interesting one is a retinoid-induced class II tumor suppressor protein.[59] The sequences of the homologous proteins are shown in Scheme 11. A noteworthy homologous stretch is in the consensus region between positions 130−135. This region contains a cysteine residue, which proved to be essential for LRAT catalysis.[58] The great sensitivity of LRAT to thiol reagents coupled to its relative insensitivity to serine reagents is consistent with the notion that LRAT might possess a cysteine residue essential for catalysis.

LRAT from human retinal pigment epithelium has cysteine residues at positions 161, 168, 182, and 208. Site-specific mutagenic studies show that C182 and C208 can be converted to alanines with little effect on activity.[60] Even the double mutant C182A/C208A is essentially fully active biochemically.[60] However, the activities of the C161A and C168A are virtually nil. Moreover, while C168S is substantially active, C161S possesses only a few percent of the activity of WT LRAT.[60] In addition, C168S is inactivated by RBA-type affinity labeling agents, C161S is unaffected.[60] It is also instructive to compare pH versus rate and K_M profiles for the mutants as compared to WT LRAT. The rate versus pH profile for WT LRAT shows two pK_A values at 8.3 and 10.8. The lower pK_A value is consistent with a cysteine residue being important for catalysis. The higher pK_A is in a range where a lysine residue might be considered. The K_M profile shows a pK at 8.7. Mutant C168S is virtually identical to WT LRAT with respect to these profiles. Therefore, the catalytic machinery required for substrate processing is left intact in this mutant. On the other hand, C161S certainly did not provide pH profiles consistent with WT LRAT. The experiments taken together are certainly consistent with LRAT being a thiol acyltransferase, and C161 may be the essential nucleophilic residue critical for catalysis. C161 also appears in a projected α-helical region of LRAT and is also in a conserved region of the family of proteins of unknown function that LRAT is homologous to (Scheme 11). It is also interesting to note that LRAT appears to be mechanistically unusual

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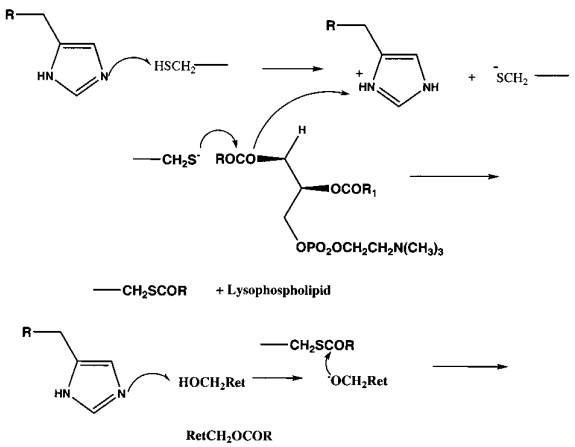
and not in the class of the typical serine lipases and acyl transferases. Whether this mechanism will define a new class of membrane-bound acyl transferases is unknown. Since so much of what is known about enzyme mechanisms is derived from studies on soluble proteins, it is perhaps not too surprising to find unusual mechanisms in the area of catalysis by membrane-bound enzymes where structural constraints are likely to be unique to this class of enzymes.

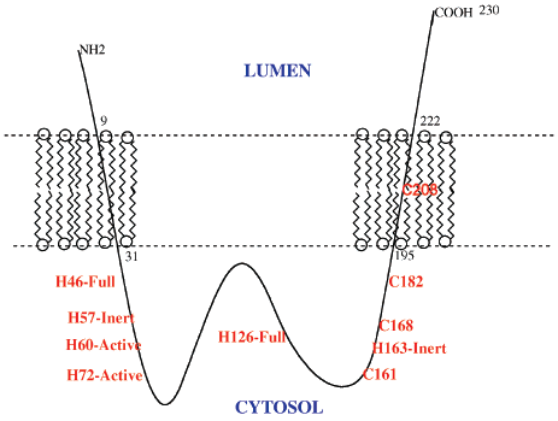
The experiments defining the catalytic role of C161 do not, of course, help in the identification of other essential amino acid residues. Given the roles of histidine residues in many protease/transferase type mechanisms, it is of interest to probe the possible importance of histidine residues in LRAT function. LRAT contains six histidine residues at H46, H57, H60, H72, H126, and H163.[58] The various glutamine for histidine mutants have been expressed in HEK-293T cells and analyzed for activity. A series of the six mutants were studied (H72Q, H60Q, H126Q, H46Q, H57Q, and H163Q). The H72Q, H60Q, H126Q, and H46Q mutants were quite active in the LRAT assay.[61] In fact, H72Q is substantially more active than WT LRAT. Therefore, H72, H60, H126, and H46 can be removed from consideration as being either catalytically or structurally important for LRAT action. The same is not true for H57 and H163, because both H57Q and H163Q are catalytically inert. The LRAT activities of these two mutants were indistinguishable from that of H293 cells transfected with empty vector and are thus inert.[61]

The experiments rule out the possibilities that H72Q, H60Q, H126Q, H46Q are catalytically important. H57 and H163, on the other hand, are important for catalytic function. It is interesting to note that only these two histidine residues are found in regions of LRAT predicted to be α-helical (Scheme 9). Moreover, C161 is the catalytically active thiol group of LRAT. This cysteine residue is then quite close spatially to H163. Since no structural information is available on LRAT, the relative spatial orientation of H57 and C161 are unknown at present, but, of course, they could be nearby in space. A possible mechanism involving the two histidine residues is provided in Scheme 12. The ping-pong kinetic mechanism for LRAT indicates that a *sn*-1 acyl moiety of lecithin is first transferred to an active-site nucleophile of LRAT, probably C161.[45,58] In ensuing steps, the lysolecithin leaves and vitamin A is bound. Finally, the acyl moiety of the acyl-enzyme intermediate is transferred to the vitamin A, generating the retinyl ester. Base catalysis is required in this kind of mechanism, and the putative involvement of the two histidine residues in base catalysis is shown in Scheme 12. One of the histidine residues could act as a base to generate the thiolate anion in the first step, which then reacts as the nucleophile attacking lecithin and generating the thioacyl intermediate. In step two, the second histidine residue could act as a general base to help abstract the HO proton of vitamin A, thus increasing its nucleophilicity and enhancing its ability to attack the acyl enzyme intermediate (Scheme 12). The validity of this hypothesis is currently being tested experimentally.

Scheme 12. Possible Role of Histidine Residues in the Catalytic Mechanism of LRAT



Scheme 13. Essential Cysteine and Histidine Residues of LRAT[a]



[a] The activities of the point mutants Q for H and S for C are indicated.

As mentioned above, LRAT shows two pK_A values at 8.3 and 10.8. The pK_A value of 8.3 is probably ascribed to C161. The pK_A of 10.3 could conceivably be attributed to a histidine residue. However, a histidine residue with such a high pK_A would have to be in an unusual micro-environment. A functionally important lysine or tyrosine residue would be more in keeping with this high pK_A value. Further experiments will define the possible roles of the lysine and tyrosine residues in catalysis. Scheme 13 provides a cartoon of LRAT and summarizes the site-specific mutagenic work.

A final issue concerning LRAT catalysis concerns its protomeric state in RPE membranes. As isolated, LRAT is a 25.3-kDa protein. However, radiation inactivation analysis in liver cells suggests a molecular weight closer to 50 kDa.[62] In fact, LRAT monomer is readily cross-linked in RPE membranes using thiol cross-linking agents.[63] The cross-linked product is fully catalytically active, suggesting the possibility that LRAT may be active as a homodimer in membranes. This possibility would reconcile the biochemical results with the radiation inactivation analysis.

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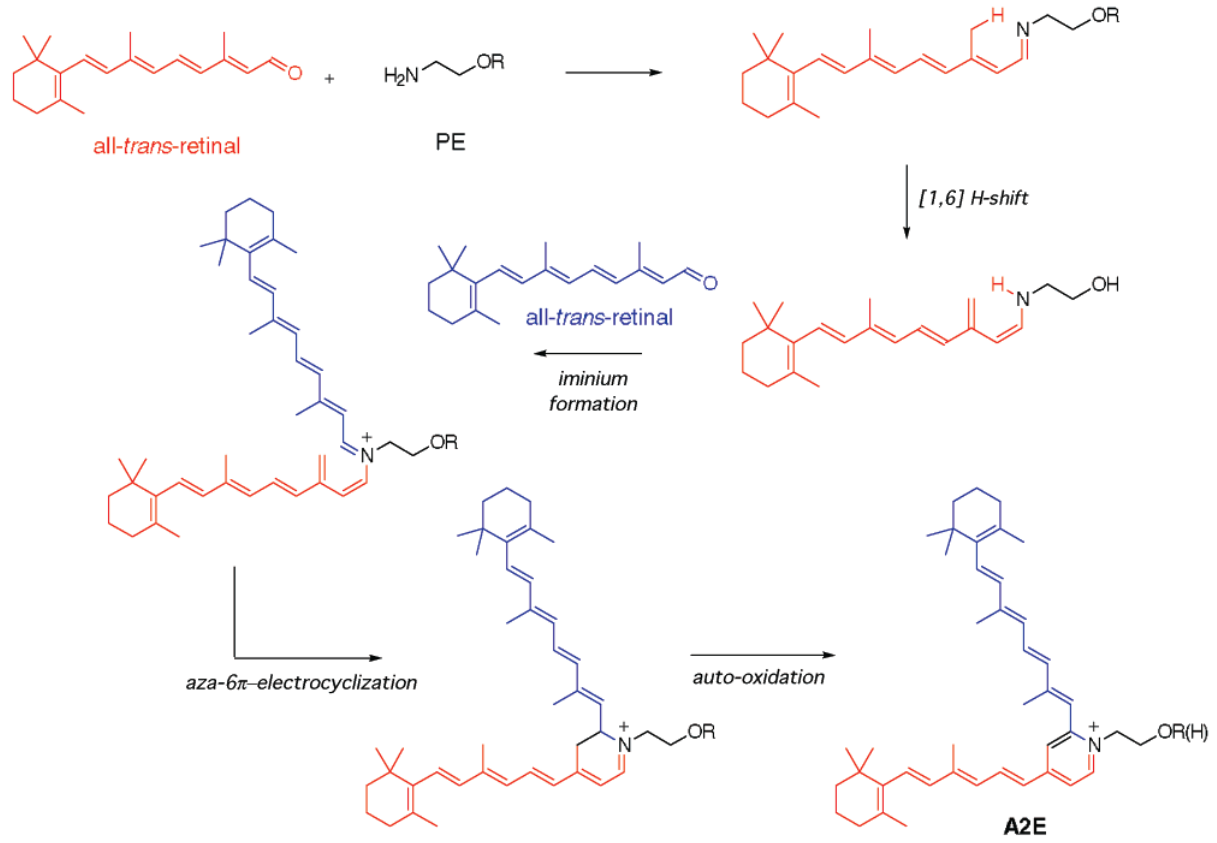
Scheme 14. Synthesis of a Retinotoxic Adduct (A2E) Formed by the Condensation of Phoshatidylethanolamine (PE) and all-*trans*-Retinal



A great deal of analysis and experimentation are still required on LRAT before a chemically coherent picture emerges on this interesting enzyme. What is clear though is that there may be many mechanistic surprises to be had as the experimentally recalcitrant minor membrane proteins are rendered susceptible to analysis. It will be interesting to see if new mechanistic paradigms are followed as a result of novel protein folding patterns necessitated by proteins having to fold in the lipid bilayer. At least in the case of LRAT, it is clear that a mechanistic pathway is chosen which would not have been predicted by analogy with analogous soluble enzymes.

LRAT clearly defines an unusual enzymatic activity that is part of the visual cycle. It is of interest to consider the remaining enzymes that constitute the visual cycle to determine whether they also may be mechanistically unusual inasmuch as they too are membrane associated. The following sections will provide background on the remaining enzymes of the visual cycle.

C. The Retinol Dehydrogenases

There are at least two different classes of retinol dehydrogenases essential in the visual cycle. The first retinol dehydrogenase is associated with photoreceptor membranes and is required to reduce the all-*trans*-retinal produced as a result of the photochemical bleaching of rhodopsin.[6,7,10−12] The second enzyme(s) is found in the RPE and is able to oxidize 11-*cis*-retinol, the product of isomerization action to generate the visual chromophore 11-*cis*-retinal.[64−66] The 11-*cis*-retinal is then delivered to the photoreceptor cells to complete the visual cycle. Physiologically important members of both classes of enzymes have now been identified, cloned, and sequenced.[65,66]

The rod all-*trans*-retinol dehydrogenase is an important enzyme in the visual cycle. This enzyme begins the regeneration pathway by reducing the all-*trans*-retinal which is hydrolyzed from bleached rhodopsin. Beyond the obvious importance of the enzyme in visual cycle function, it is important for at least two other reasons. First, and most importantly, the reductive step in question detoxifies the highly reactive all-*trans*-retinal. Second, evidence exists at least in rodents, to suggest that the rate of all-*trans*-retinal reduction may be rate limiting in the visual cycle.[67]

The detoxifying role of all-*trans*-retinol dehydrogenase has only recently been elucidated. All-*trans*-retinal is generated in the disk membranes where rhodopsin is located. The phospholipids of these membranes are rich in phosphatidylethanolamine (PE) which can engage in Schiff base formation with all-*trans*-retinal (Scheme 14).[68] Before the irreversible steps leading to A2E occur, initial reversible Schiff base formation occurs. This Schiff base is normally labile enough via hydrolysis so that in the presence of the extra-diskal all-*trans*-retinol dehydrogenase,

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all-*trans*-retinol (vitamin A) is rapidly generated.[7] Whether the Schiff base is hydrolyzed catalytically or not is unclear at this stage. The question emerges as to how the PE-retinal Schiff base on the inner leaflet of the disk membranes can be further processed by all-*trans*-retinol dehydrogenase, as the latter resides outside the disk membranes. It has recently been found that a disk membrane associated ABCR transporter exists that can pump the Schiff base out of the disks into the cytoplasm as a consequence of hydrolyzing ATP.[69−72] This movement of the PE-retinal Schiff base out from the luminal to cytoplasmic side where it can be processed by the dehydrogenase is very important because the PE-Schiff base can undergo a series of reactions that render the PE-retinal adduct irreversible as shown in Scheme 14.[73−75] When this adduct A2E is taken up by the RPE by a phagocytic process, it is oxidized to form a major constituent of a highly retinotoxic mixture referred to as the lipofucins.[72−75] These compounds cannot be further metabolized in the RPE, and they build up with time and are associated with retinal degenerative disorders and blindness.[75−78] It is interesting to note that mutational defects in the ABCR transporter that decrease its efficiency also enhance retinal degeneration because they have the effect of decreasing turnover of the lipofuscin precursors.[75]

As mentioned above, the relevant visual all-*trans*-retinol dehydrogenase has been cloned.[7] The enzyme is a member of the short chain alcohol dehydrogenase family, is most closely related to 17-β-hydroxysteroid dehydrogenase, and favors NADPH over NADH.[7] The enzyme is membrane associated and is stereoselective with respect to the reduction all-*trans*-retinal versus 11-*cis*-retinal.[7] There is little doubt now that the biologically relevant all-*trans*-retinol dehydrogenase of the visual system has now been identified both in rods and cones.

The RPE 11-*cis*-retinol dehydrogenase is, of course, also essential in the completion of the visual cycle, and this enzyme has also been recently cloned and sequenced.[64,65] This 33-kDa protein is also membrane bound, probably through its N- and C-termini.[64,65] The enzyme is stereospecific for 11-*cis*-retinol and preferentially uses NAD as its cofactor.[63,64] Like its all-*trans*-retinol dehydrogenase counterpart, 11-*cis*-retinol dehydrogenase is also related to 17-β-hydroxysteroid dehydrogenase in sequence.[64,65] A further interesting aspect of 11-*cis*-retinol-dehydrogenase is the fact that it associates with a major RPE membrane protein referred to as p63[26] or RPE 65[79] which appears to play an important, although thus far undefined role, in visual cycle function.[80] It has been suggested that this protein may be a component of the membrane receptor for retinol binding protein,[26] although no firm evidence exists for this possibility.

An interesting aspect of the molecular function of 11-*cis*-retinol dehydrogenase (RDH) relates to its spatial orientation.[81] Freshly prepared RPE microsomes containing RDH were treated with various proteases with little effect on RDH, while detergent solubilized RDH is readily digested.[81] These and related experiments strongly suggest that the catalytic domain of RDH is lumenal rather than cytoplasmic.[81] This in turn suggests that other cytoplasmic binding proteins, such as cellular retinal binding proteins (CRALBP),[82] are unlikely to physiologically interact with RDH. Retino(a)ls themselves can, of course, readily transverse membranes so that there is not necessarily a barrier here for the effective percolation of 11-*cis*-retinal(ol) in and out of the cytoplasm. The 11-*cis*-retinal must eventually be delivered to photoreceptors to complete the visual cycle. Although, as in the case of the ABCR transporter for all-*trans*-retinal in disk membranes, a similar RPE microsomal transporter for 11-*cis*-retinal is also possible.

Interestingly, RDH has been implicated in visual disorders. For example, mutations in RDH have been found to be a major cause of a congenital night-blindness disorder known as fundus albipunctatus.[83] A mouse model of an RDH knockout showed little in the way of visual deficits suggesting that either the wrong enzyme was knocked out or that multiple RDH activities exist in the mouse.[83] Of pharmacological interest is the fact that 13-*cis*-retinoic acid, a drug used in the treatment of acne and other skin disorders, can cause night blindness as a toxic side effect.[84] A possible reason for this resides in the fact that 13-*cis*-retinoic acid is a competitive inhibitor of RDH and could therefore decrease the rate of rhodopsin regeneration.[84]

D. Other Enzymatic Activities of the Visual Cycle

Of course, the major enzymatic activity of the visual cycle that still remains unidentified is the isomerohydrolase. This activity was first identified in in vitro experiments in 1987.[13] In fact, this was the first time 11-*cis*-retinoid biosynthesis had ever been demonstrated outside the living eye. There are some aspects of isomerohydrolase activity that are understood, however. The enzyme is found largely, if not entirely, in the RPE and is membrane bound.[13] The isomerohydrolase reaction is, as expected, reversible in vitro.[33] The enzyme is strongly feedback inhibited by 11-*cis*-retinoids,[86] which explains the apparent sluggish activity of the enzyme in the absence of retinoid binding proteins capable of sequestering the 11-*cis*-retinoids (Scheme 15).[85] Even the nonspecific bovine serum albumin will do here, although CRALBP, a RPE specific 11-*cis*-retinol(al) binding protein,[82,86−88] is more effective on a molar basis, but does not increase the Vmax for the isomerization reaction.[85] The role of the binding proteins is to remove 11-*cis*-retinol from the enzyme's active-site to allow for further processing.

The specificity of the inhibition by 11-*cis*-retinoids has been further investigated.[63] It is clear that added 11-*cis*-retinol, but not 13-*cis*-retinol (**5**, Scheme 16), potently inhibits the isomerohydrolase, suggesting that the inhibition process is specific.[63] The specificity of inhibition was further explored here. Since added retinols are esterified by endogenous LRAT, it is unclear whether the inhibition observed with 11-*cis*-retinol is due to this molecule, or retinyl esters formed from it. Studies with 11-*cis*-retinal as putative inhibitor can decide this issue. In fact, 11-*cis*-retinal

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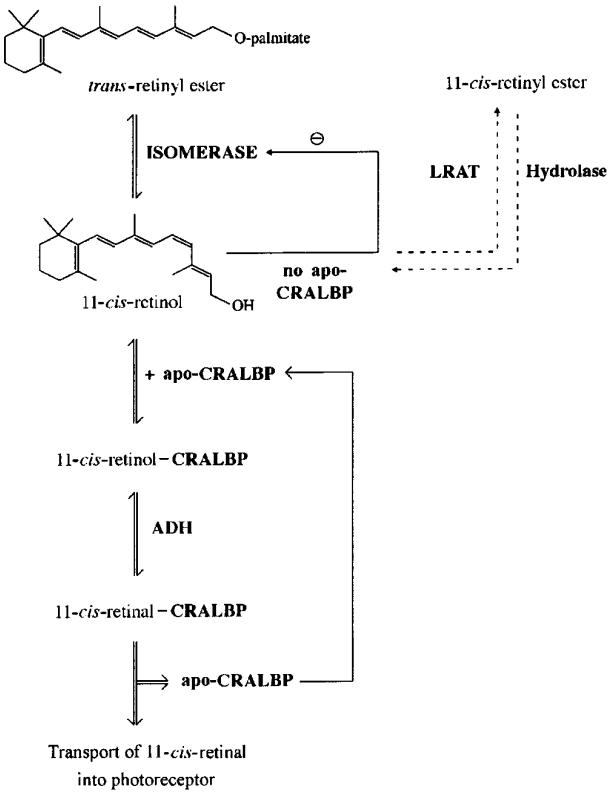
Scheme 15. Feedback Inhibition of Isomerization by 11-*cis*-Retinoids



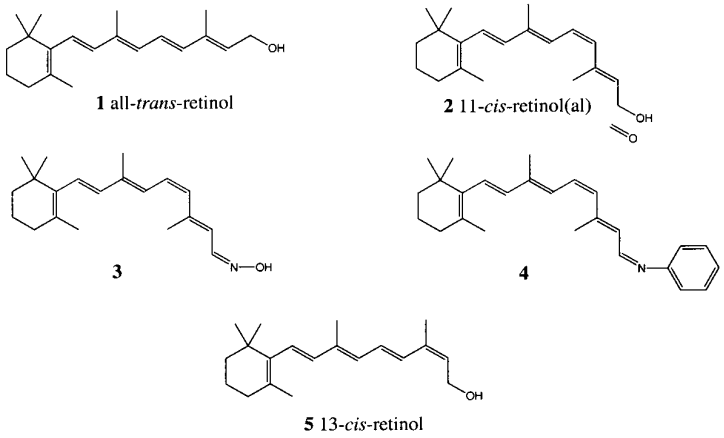
is approximately equipotent with 11-*cis*-retinol with respect to isomerohydrolase inhibition. Since redox chemistry is possible in the membranes used (the RPE microsomal membranes contain a large number of enzymes other than the isomerohydrolase, including 11-*cis*-retinol dehydrogenase, and LRAT inhibition studies using 11-*cis*-retinol(al) were carried out in the presence of 4-methylpyrazole, a potent inhibitor of ocular retinol dehydrogenases,[89] with no difference in results. Therefore, 11-*cis*-retinal itself inhibits the enzyme as well as 11-*cis*-retinol. The specificity of the 11-*cis*-retinol(al) mediated inhibition is illustrated by the observation that neither 11-*cis*-retinal oxime 3 (Scheme 16) nor aniline Schiff base 4 (Scheme 16) proved to be inhibitors of the enzyme.

While retinyl esters could not be directly studied because of retinyl ester hydrolytic activity present in the membrane system used, results with the oxime and Schiff base render it unlikely that 11-*cis*-retinyl esters would be capable of potently inhibiting the isomerohydrolase. Thus, the activity of isomerohydrolase is limited by product formation. In darkness, where 11-*cis*-retinal is not required, its precursor 11-*cis*-retinol is formed only sluggishly. In the light, however, 11-*cis*-retinol is rapidly synthesized and oxidized by 11-*cis*-retinol dehydrogenase to form chromophore.

Virtually all of the information presently available on the isomerization process comes from in vitro and in vivo experiments. The immense practical difficulties in isolating the enzyme(s) have to do with several factors. First and foremost, it is a minor membrane-bound enzyme, and the factors that make the identification and purification of these proteins particularly vexing have already been discussed. Further compounding difficulties here include the fact that the enzyme is relatively unstable in detergent and is not readily inhibited by group-specific reagents. The latter issue makes it difficult to rationally design affinity labeling agents of the enzyme possibly useful for its identification. Finally, since this enzyme is a unique one, after all 11-*cis*-retinoids are only synthesized in the eye, cloning by homology is likely to be fruitless. This approach has proved to be very useful in the cloning of the retinol dehydrogenases of the visual system, for example.[7] Here investigators possessed a wealth of sequence information on homologous NAD(P) linked dehydrogenases to guide the cloning efforts. None of this is to say though that interesting candidates for isomerohydrolase do not exist. RPE65,[80] also known as p63,[26] has been presented as one.[80]

This interesting protein, which is a major membrane-associated protein found in the RPE, appears to be essential for 11-*cis*-retinoid biosynthesis from mice knockout studies.[80] Mutations in this protein have also been implicated in human visual degenerative disorders.[90−92] However, it seems to be more essential for rod regeneration than for cone regeneration.[80] Certainly there is nothing to say that 11-*cis*-retinoid regeneration in rods and cones may not be

Scheme 16. Analogues Used to Probe the Specificity of the Feedback Inhibition Process



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different. Importantly, though, there is no direct evidence that RPE65/p63 has anything to do with isomerization per se. In fact, the stripping of RPE65/p63 from RPE membranes does not appear to strongly affect the abilities of these membranes to synthesize 11-*cis*-retinoids from added vitamin A.[63] However, it is possible that the extent of isomerization by RPE membranes is not linear with the amounts of RPE65/p63 present. This protein is clearly very important in the visual cycle and may define a novel component in the cycle. It could be a novel retinoid binding protein, a receptor for a retinoid binding protein, or a protein essential for the mobilization of the insoluble retinyl esters. Whatever the actual role of RPE65/p63 is, it is bound to be interesting and important for visual cycle studies.

Mobilization of retinyl esters has been given scant attention over the years. Retinyl esters themselves are highly insoluble in aqueous milieu and undergo negligible rates of intermembranous transfer.[22] Thus, it would not be unreasonable to think that proteins would have evolved to process these molecules. Already there is some evidence to suggest that stereoselective retinyl ester esterase activities are present in the retina/RPE.[39,40] These activities have not yet been characterized, so it is difficult to decide whether they are specific for retinyl esters or are nonspecific esterases. As previously mentioned, a stereospecific 11-*cis*-retinyl ester hydrolase could be of central importance in thermodynamically driving the visual cycle.

III. Conclusions

Although the biochemical outlines of the vertebrate visual cycle are largely in place, a great deal of understanding is still lacking both at the integrative level and at the level of simply describing the components. On the surface, not being in a position to describe the components of the cycle even though it has been almost 15 years since the first description of an in vitro system capable of carrying out de novo 11-*cis*-retinoid biosynthesis seems surprising.[13] However, it is not surprising when one considers the immense difficulties in place when attempting to identify integral membrane-bound enzymes, especially if they are minor components. Unfortunately, most of the enzymes of the visual cycle appear to be minor integral membrane proteins, and, as expected, their identification and purification has been slow. As mentioned in the introduction, the issue of identification and purification of integral membrane enzymes represents an enormous problem in biology. While many of these enzymes will doubtless operate by rules similar to those established for soluble enzymes, many will not. LRAT is a good example of this. Even though the LRAT sequence appeared in the human genome data bank, its function would have remained obscure had not partial sequence information been obtained on it first. New techniques will need to be developed in the area of what is referred to as functional proteomics to address the huge issue of minor membrane-bound proteins. We believe that the biotin labeling techniques developed for the identification of LRAT will comprise at least part of the solution. With respect to the vertebrate visual cycle, there are many outstanding problems of enzyme identification remaining.

The most outstanding problem seems to be in the identification and mechanistic analysis of the isomerohydrolase. Is this one enzyme or multiple enzymes? Analysis of the visual cycle identifies two great thermodynamic driving forces for the production of 11-*cis*-retinoids. In the photoreceptors, of course, it is the binding of 11-*cis*-retinal to opsin, while in the RPE it is the hydrolysis of retinyl esters. Here, the coupling of retinyl ester hydrolysis to 11-*cis*-retinol biosynthesis needs to be clarified. Specifically, it will be important to understand the possible role(s) of specific 11-*cis*-retinyl ester hydrolysis in the pathway. Another important problem is to reveal the functional role of RPE65/p63 in visual cycle function. Is it an integral part of the isomerization pathway as mouse knockout experiments would imply?[80] Possibly, it is involved in the mobilization of the insoluble retinyl esters, or it may even have a structural role to play in an isomerohydrolase multienzyme complex.

The enzymes of the visual cycle have been the subject of this review, but retinoid binding proteins certainly loom large in the operation of the visual cycle. Since many of these are soluble proteins, they have been purified and studied in some detail. IRBP is a major interphotoreceptor protein that is thought to play an important role in shuttling retinoids between the RPE and photoreceptors.[14−21] However, recent experiments on IRBP knockout mice suggest that there may be alternate retinoid binding proteins available in the absence of IRBP.[20,21] CRALBP is the only stereospecific (for 11-*cis*-retinol(al))retinoid binding protein thus far identified.[86−88] Its precise role is somewhat unclear, but it does appear to be physiologically of consequence from studies on human retinal degenerative disorders.[93] An important role for this protein may be in the removal of 11-*cis*-retinol(al) from inhibiting isomerohydrolase.[85] Its earlier stated role of enhancing the rate of 11-*cis*-retinol oxidation by RDH seems remote given the luminal localization of the dehydrogenase.[83] Certainly there may be other retinoid binding proteins to be yet discovered, especially if they are membrane associated. Overall, these binding proteins must play an important role in the vectorial delivery and chemical protection of the chemically unstable retinol(als). They may also play an important role in the regulation of the visual cycle as implied by the ability of CRALBP to activate isomerohydrolase by relieving the inhibition of the enzyme by 11-*cis*-retinoids. The issue of visual cycle regulation remains a large and important question.

Issues to be confronted here involve how the biosynthesis of 11-*cis*-retinal may be influenced by light intensity. Certainly, the fluxes of 11-*cis*-retinoids through the system need to be vastly accelerated in the light. It is already known that 11-*cis*-retinoid biosynthesis in the dark is a very sluggish affair.[13,35,85] Part of this diminished rate is certainly due to feedback inhibition of isomerization by accumulating 11-*cis*-retinoids. However, it is certainly possible that other regulatory mechanisms and other pro-

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cesses exist to compensate for the increased requirement for chromophore during light exposure. Along these lines it is reasonable to ask whether there may be more than one route to 11-*cis*-retinoid biosynthesis in vertebrates.

It is interesting to consider why nature has chosen an enzymatic rather than a photochemical route to the biosynthesis of 11-*cis*-retinoids. After all, light is available in the eye to drive the thermodynamically uphill isomerization. Part of the reason probably is related to the necessity for 11-*cis*-retinal synthesis under conditions of very low light illumination. Moreover, control is best exerted on an enzymatic process. However, what about under conditions of high light illumination? Recent provocative studies on the so-called RPE retinal G protein-coupled receptor (RGR) protein(s) may address this issue.[94] This opsin-like protein facilitates the photoisomerization of all-*trans*-retinal to 11-*cis*-retinal and could provide a ready source of chromophore under conditions of high light flux.[94]

Clearly, there are many issues of the basic science of visual cycle function that need to be addressed in the future. How this basic science knowledge will inform our understanding of the underlying causes of diseases of vision is a whole other topic of immense importance.

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