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# Membranes as the Energy Source in the Endergonic Transformation of Vitamin A to 11-*cis*-Retinol

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**The eye needs to biosynthesize 11-*cis*-retinoids because the chromophore of rhodopsin is 11-*cis*-retinal. The critical metabolic step is the endergonic isomerization of free all-*trans*-retinol (vitamin A) into 11-*cis*-retinol. This isomerization process can take place in isolated membranes from the retinal pigment epithelium in the absence of added energy sources. Specific binding proteins probably do not serve as an energy source, and since all of the reactions in the visual cycle are shown here to be reversible, trapping reactions also do not participate in the isomerization reaction. One previously unexplored possibility is that the chemical energy in the bonds of the membrane itself may drive the isomerization reaction. A group transfer reaction is proposed that forms a retinyl ester from a lipid acyl donor and vitamin A. This transfer can drive the isomerization reaction because the all-*trans*-retinyl ester is isomerized directly to 11-*cis*-retinol. Thus, the free energy of hydrolysis of the ester is coupled to the thermodynamically uphill trans to cis isomerization. The prediction of an obligate C–O bond cleavage in the vitamin A moiety during isomerization is borne out. Although the natural substrate for isomerization is not known, all-*trans*-retinyl palmitate is processed in vitro to 11-*cis*-retinol by pigment epithelial membranes.**

THE ISOMERIZATION OF FREE ALL-*trans*-retinol (vitamin A) into 11-*cis*-retinol in the retinal pigment epithelium provides the visual chromophore and closes the visual cycle. In the absence of this process vertebrate vision would be impossible, because 11-*cis*-retinoids are produced only in the eyes of image-forming animals and are not dietary constituents. The 11-*cis*-retinoids are approximately 4 kcal/mol higher in free energy than their all-*trans* congeners, and thus account for only 0.1% of the equilibrium mixture (*1*). Yet in an animal adapted to darkness, 11-*cis*-retinoids can account for ~75% of the retinoids (*2*). Hence, an energy-requiring step seems to be necessary.

A membrane fraction from the retinal pigment epithelium has been isolated that can process exogenous all-*trans*-retinol to 11-*cis*-retinol, 11-*cis*-retinal, and 11-*cis*-retinyl esters (*3*). The process is saturable with a Michaelis constant ($K_m$) of 0.5 $\mu M$ and a basal maximal velocity ($V_{max}$) of 5 pmol hour$^{-1}$ mg$^{-1}$ of protein, and 11-*cis*-retinoids accumulate to account for up to 40% of the total retinoids (*4*). The conversion occurs in the absence of added energy sources such as adenosine 5′-triphosphate, guanosine 5′-triphosphate, and palmitoyl coenzyme A. Likewise, the addition of these agents does not stimulate 11-*cis*-retinoid production, suggesting that soluble cofactors are not required for the isomerization (*4*) and that the energy source is membrane associated.

Three possible mechanisms could account for the formation of the high-energy retinoids. (i) An irreversible reaction removes the 11-*cis*-retinoids as they are formed. (ii) Specific binding proteins sequester the 11-*cis*-retinoids. (iii) An energy-transducing isomerization reaction uses a membrane constituent as the thermodynamic energy source. We now show that only the last alternative can account for the experimental results, and we provide a mechanism for this process.

We investigated whether the membranes act catalytically in the isomerization reaction or have a limited capacity to form 11-*cis*-retinoids. A membrane preparation was incubated with saturating all-*trans*-retinol until no further 11-*cis*-retinoid accumulated. A control membrane preparation was incubated in the absence of all-*trans*-retinol. The two samples were then briefly irradiated with ultraviolet (UV) light and washed with solutions containing bovine serum albumin (BSA) to remove the remaining retinoids. The UV illumination back isomerizes and eliminates the 11-*cis*-retinoids without di-

**Table 1.** The esterification-deesterification reactions are reversible. Amphibian (*3*) or bovine (*11*) pigment epithelial membranes (1 ml at a protein concentration of 2 to 3 mg/ml) were incubated with [11, 12-$^3$H]all-*trans*-retinol (Amersham) or its 11-*cis* congener (0.01 mCi at a concentration of 1 $\mu M$ in 50 $\mu$l of 10% BSA) for 15 min at 25°C. The mixtures were then centrifuged at 200,000*g* for 15 min at 4°C, and the pellets were washed and suspended in the original volume of buffer (*3*). Greater than 80% of the radioactivity remained associated with the membranes. One-half of the membrane preparation was removed, and the retinoid composition was analyzed by standard methods (*3*). In the case of the amphibian, the other half of each membrane preparation was incubated for 3 hours at 25°C with 25 $\mu$l of 10% BSA and 50 $\mu$l of a solution containing $NAD^+$ and $NADP^+$, each at a concentration of 10 m$M$, before retinoid analysis. In the bovine case, the membrane preparations were incubated for 1 hour at 37°C with 25 $\mu$l of 10% BSA and 50 $\mu$l of 10 m$M$ each of $NAD^+$ and $NADP^+$ for 1 hour at 37°C before analysis of the retinoids. All quoted errors are SEM with $n$ = 3 to 5.

| Membrane preparation | Retinoid composition (%) | | |
|---|---|---|---|
| | Retinol | Retinal | Retinyl ester |
| Amphibian | | | |
| All-*trans*-retinol | 5.3 ± 2.0 | 4.3 ± 0.4 | 90.3 ± 2.0 |
| All-*trans*-retinol, $NAD^+$, and $NADP^+$ | 5.4 ± 1.7 | 49.5 ± 4.6 | 45.0 ± 3.1 |
| Bovine | | | |
| All-*trans*-retinol | 6.3 ± 2.2 | 3.9 ± 3.5 | 89.9 ± 1.3 |
| All-*trans*-retinol, $NAD^+$, and $NADP^+$ | 6.0 ± 3.0 | 49.7 ± 4.1 | 44.4 ± 7.7 |
| 11-*cis*-retinol | 12.2 ± 0.0 | 3.9 ± 0.1 | 84.0 ± 0.1 |
| 11-*cis*-retinol, $NAD^+$, and $NADP^+$ | 10.9 ± 0.6 | 71.1 ± 0.8 | 17.5 ± 0.1 |

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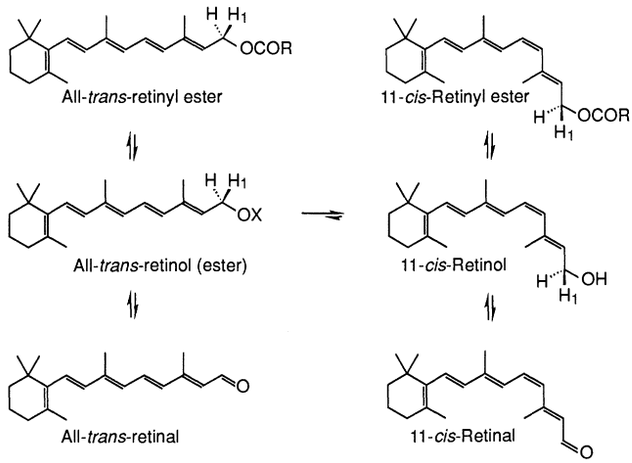
rectly affecting the isomerizing capability of the membranes. The membrane samples were then incubated with saturating [11,12-$^3$H]all-*trans*-retinol again until no further 11-*cis*-retinoid accumulated. The membranes treated with all-*trans*-retinol had an average of 55 ± 11% (mean ± SE; $n$ = 3) of the capacity of the control membranes to form 11-*cis*-retinoids. This result suggests that a nonreplenishable energy source can be depleted in the membranes and that specific binding proteins are probably not involved in the isomerization. This view is consistent with the observation that unbound 11-*cis*-retinyl esters accumulate both in vivo (*2*) and in vitro (*3*). Because these esters make up the major storage form of retinoids in the eye, their being found uncomplexed is inconsistent with a role for binding proteins as a driving force.

An irreversible reaction in the visual cycle could drive the isomerization process toward 11-*cis*-retinoid formation by not allowing equilibrium to be achieved. In addition to performing the isomerization reaction, the retinal pigment ephithelial membranes can further process the retinols both by oxidation to the retinals and by esterification to the retinyl esters (*3*). In fact, the membranes retain all of the vitamin A processing activities that are found in the living eye and that constitute the visual cycle. The oxidation reactions can be manipulated by varying the ratio of oxidized to reduced nicotinamide adenine dinucleotide ($NAD^+$ to NADH) or nicotinamide adenine dinucleotide phosphate ($NADP^+$ to NADPH) (*3*). Thus we can determine if the esterification reaction is reversible. Membranes were incubated with [11,12-$^3$H]all-*trans*-retinol in the presence or absence of $NAD^+$ and $NADP^+$ (Table 1). Addition of the $NAD^+$-$NADP^+$ mixture to either amphibian or bovine membranes after the esters were formed increased the size of the retinal pool at the expense of the retinyl esters. This result shows that the esterification-deesterification cycle is reversible and that no irreversible trapping reaction occurs. Although it has been assumed that the liberation of the retinols from their ester counterparts is mediated by one or more hydrolases, this remains unproved (*5*). Esterification of retinol both in the liver (*6*) and in the retinal pigment epithelium (*7*) occurs through transfer of an acyl group from the 1 position of an endogenous phospholipid. This transesterification reaction is, in principle, reversible.

To determine if the isomerization reaction itself is reversible, chiral retinols had to be used because of the spontaneous isomerization of 11-*cis*- to all-*trans*-retinol. The enzymatic isomerization occurs with inversion of stereochemistry at the C-15 prochiral methylene hydroxyl group (Scheme 1) (*8*), whereas chemical isomerization occurs with retention of stereochemistry at this position. Membranes were incubated with [15(*S*)-$^{14}$C,$^3$H]11-*cis*-retinol, and the all-*trans*-retinol that was formed was examined for the amount of inversion that had occurred. This latter determination was made with *pro*-R–specific horse liver alcohol dehydrogenase (Table 2) (*9*). The results show that substantial enzyme-catalyzed back isomerization occurred, which would not have been expected if only the extent of isomerization had been determined. Thus, the isomerization reaction is reversible. As expected, the thermal isomerization occurred with retention of configuration (Table 2). In a separate experiment beginning with [11,12-$^3$H]all-*trans*-retinol under the same conditions, 31% (of total retinols) 11-*cis*-retinol and 24% (of total esters) 11-*cis*-retinyl esters were formed. This result suggests that the forward trans to cis isomerization is approximately threefold faster than the reverse reaction. Incubation of the membranes with [15(*S*)-$^{14}$C,$^3$H]all-*trans*-retinol also showed a small (<10%) amount of inversion in the starting material, demonstrating that enzymatic processing to 11-*cis*-retinol occurs and is followed by chemical back isomerization.

**Scheme 1.** Reactions of the cycle are reversible. X = H, all-*trans*-retinol; or X = COR, all-*trans*-retinyl esters.



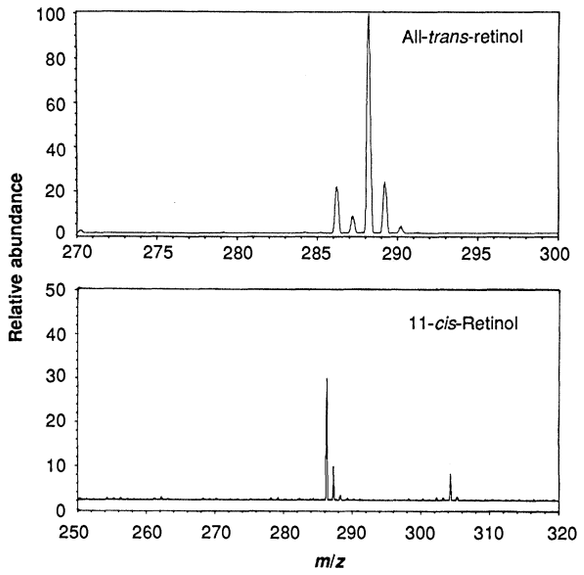




**Fig. 1.** C–O Bond cleavage accompanies isomerization. All-*trans*-retinol labeled with $^{18}$O was prepared in an amber vial stoppered under $N_2$ by incubating all-*trans*-retinal with 1 ml of $H_2{}^{18}O$ and 2 ml of dioxane containing 1 mg of *p*-toluenesulfonic acid and 10 mg of butylated hydroxytoluene. The mixture was stirred for 18 hours at 50°C. A sample was removed for photoisomerization to provide 11-*cis*- and 13-*cis*-retinal isomers. The $^{18}$O-labeled retinals were reduced with $NaBH_4$ and separated by HPLC. Two different syntheses were performed, producing [$^{18}$O]all-*trans*-retinol containing 97.1% $^{18}$O and 95.6% $^{18}$O as determined by field desorption mass spectroscopy. Membranes from bovine pigment epithelia were prepared as previously reported (*11*). Typically, 150 bovine eyecups were used for each incubation with [$^{18}$O]all-*trans*-retinol. The membranes were homogenized in 8 ml of 50 m*M* potassium phosphate buffer (*p*H 7.4) containing 3 ml of 20% bovine serum albumin (BSA). The mixture was kept on ice and irradiated with UV light for 10 min while shaking to destroy any endogenous retinoids. The membranes were centrifuged at 150,000*g* at 4°C, washed, and resuspended in 35 ml of phosphate buffer containing 3% BSA. The [$^{18}$O]all-*trans*-retinol (20 μ*M* final concentration) was added in the dark, and the membrane preparations were placed under $N_2$ and incubated at 38°C for between 2.5 and 5 hours in the dark. The samples were then placed on ice, and 40 ml of ice-cold methanol were added. The retinoids were extracted into *n*-hexane, which was then filtered, dried, and processed by HPLC (*3*). The various retinol isomers were collected and rechromatographed before analysis by field desorption mass spectroscopy. Samples stored at −80°C showed no loss of $^{18}$O over a period of several weeks. Typical mass spectroscopic data for the reisolated [$^{18}$O]all-*trans*-retinol and the enzymatically formed 11-*cis*-retinol are shown.

These experiments taken together show that the reactions of the visual cycle are reversible (Scheme 1) and help explain why even extensive dark adaptation in vivo never drives the all-*trans* to 11-*cis* conversion to near completion.

As for the third possible mechanism for the formation of 11-*cis*-retinoids, it is not immediately apparent how the chemical energy in the bonds of the membrane might be used to drive the isomerization reaction. One possibility involves a group transfer reaction (Scheme 2) in which vitamin A is first esterified to form an active ester, which is then isomerized by the indicated mechanism to form 11-*cis*-retinol. This latter isomerization reaction can be broken down into two partial reactions (Scheme 2) that would sum favorably. Thus, an all-*trans*-retinyl ester would be isomerized by a mechanism that couples the free energy of hydrolysis of the ester to the isomerization reaction. For example, the free energy of hydrolysis of an acyl ester group is in the range of −5 to −10 kcal/mol (*10*), which is more than enough energy to overcome the 4 kcal/mol energy difference between an all-*trans*- and an 11-*cis*-retinoid (*1*). The energy would originate in the chemical energy of acyl groupings of the phospholipid. It might be expected that a specific phospholipid type would be involved, designated as lipid OX in Scheme 2. It is already known that the 1-acyl groups of specific ocular membrane phospholipids can be transferred to retinol to produce retinyl esters (*6, 7*).

One direct test for a mechanism of the type shown in Scheme 2 is to determine if the oxygen atom of the all-*trans*-retinol is lost during the isomerization reaction. The fate of the $^{18}O$ atom of [$^{18}O$]all-*trans*-retinol [mass to charge ratio (*m/z*) of 288] can be followed by field desorption mass spectroscopy during the isomerization reaction (Fig. 1). If C–O bond cleavage is obligate during the isomerization reaction, then the 11-*cis*-retinol initially formed should lose the $^{18}O$ atom. Indeed, the 11-*cis*-retinol that formed had lost virtually all of its $^{18}O$. The small 288 peak found comes largely from the natural abundance contribution of unlabeled retinol (*m/z* of 286). In a series of six separate experiments, a net retention of 2.5% of $^{18}O$ was found in the isolated 11-*cis*-retinol compared with the all-*trans*-retinol. At the same time, there was virtually no loss in the reisolated all-*trans*-retinol. The formed 11-*cis*-retinol did not readily lose its label, as indicated by separate experiments showing that under the same incubation conditions [$^{18}O$]11-*cis*-retinol retained approximately 60% of its $^{18}O$. It is likely that the enzymatically formed 11-*cis*-retinol had retained none of its label, and that the 2.5% retention observed came from other sources. An approximately 20-fold excess of [$^{18}O$]all-*trans*-retinol was used in these experiments, and the spontaneous chemical isomerization of this material to 11-*cis*-retinol, which occurs without loss of label, could provide up to 4% of label retention. Also, the 11-*cis*-retinol and spontaneously formed 13-*cis*-retinol were not completely separated by high-performance liquid chromatography (HPLC), and hence some cross-contamination was inevitable, resulting in an increase in the amount of label in the 11-*cis*-retinol peak. Thus, these results show that a C–O bond cleavage occurs during the isomerization reaction and are thus consistent with a mechanism of the type shown in Scheme 2. The results are also in agreement with the inversion of stereochemistry at C-15, which occurs concomitantly with isomerization

**Table 2.** Isomerization of [15(*S*)-$^{14}C$,$^{3}H$]11-*cis*-retinol. Washed bovine pigment epithelial membranes (4 ml at a protein concentration of 1 mg/ml) were incubated for 1 hour at 37°C with 1 μ*M* [15(*S*)-$^{14}C$,$^{3}H$]11-*cis*-retinol [containing 0.01 mCi of $^{14}C$ ($^{3}H/^{14}C$ = 14.7)] and 0.2 ml of 10% BSA. Approximately 40% of the radioactivity was found to be in the retinol pool and the remaining 60% in the retinyl ester pool. Control incubations with heat-denatured membranes were also performed. The retinoids were isolated by HPLC (*3*), diluted with cold standards, and subjected to oxidation with *pro*-R–specific horse liver alcohol dehydrogenase in 0.1*M* glycine–sodium hydroxide buffer (*p*H 10.0) in the presence of 0.01% Tween 80 (*8*). Retinals were extracted as retinal oximes and their $^{3}H/^{14}C$ ratios were determined (*8*). In the case of the retinyl esters, the purified materials were collected from the HPLC and hydrolyzed with 5% potassium hydroxide in methanol for 10 min at 0°C to produce the corresponding retinol isomers. The latter were purified and processed as above to determine their $^{3}H/^{14}C$ ratios. All quoted errors are SEM with $n$ = 3 to 5.

| Condition | Retinol | | | Retinyl esters | | |
|---|---|---|---|---|---|---|
| | All-*trans* (%) | Retention of $^{3}H$ (%) | | All-*trans* (%) | Retention of $^{3}H$ (%) | |
| | | 11-*cis* | All-*trans* | | 11-*cis* | All-*trans* |
| Control | 13.1 ± 4.4 | 105.5 ± 2.1 | 102.5 ± 3.5 | | | |
| Membrane | 13.6 ± 4.7 | 110.6 ± 5.7 | 29.4 ± 16.9 | 21.7 ± 1.1 | 108.3 ± 3.5 | 23.9 ± 3.5 |

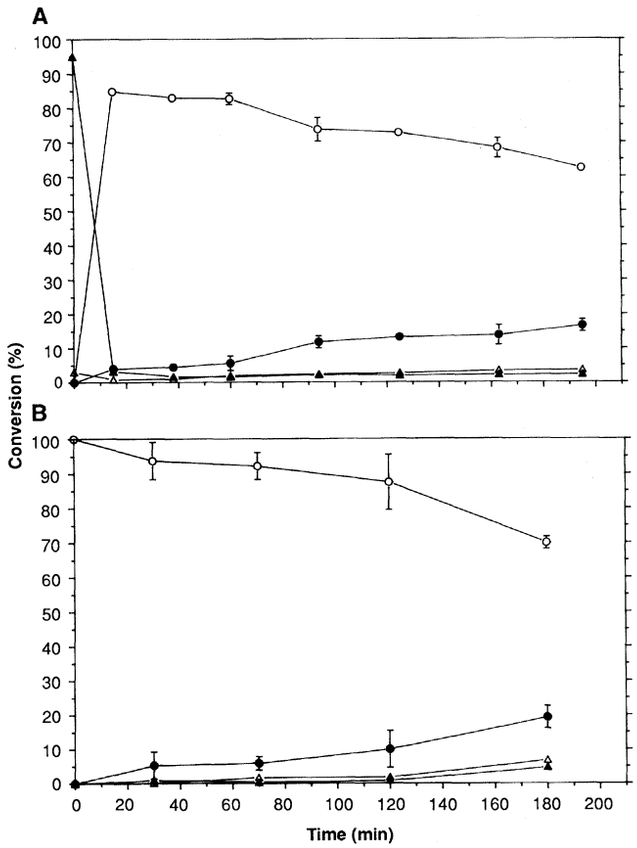
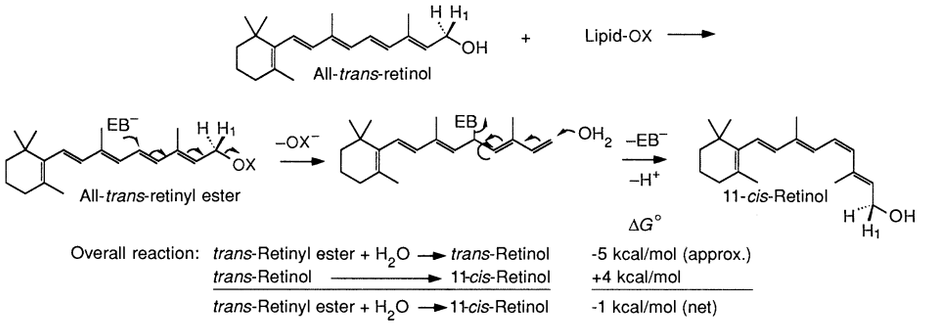



**Fig. 2.** Processing of vitamin A and all-*trans*-retinyl palmitate by irradiated pigment epithelial membranes. (**A**) Bovine pigment epithelial membranes were irradiated and washed as described in the legend to Fig. 1. The membranes (2 mg/ml) were incubated with 0.05 μ*M* [11,12-$^{3}H$]all-*trans*-retinol (50 Ci/mmol, Amersham) and BSA as previously described (*3*). In the absence of membranes no 11-*cis*-retinol was formed. At the indicated times the retinoids were extracted with methanol and hexane (*3*) and analyzed by HPLC (*3*). Small amounts of the retinals (<5%) were also found after incubation (*3*). (**B**) Membranes were incubated with synthetic [11,12-$^{3}H$]all-*trans*-retinyl palmitate (0.3 μ*M*, 5 Ci/mmol) (*3*). At the indicated times, samples were removed, and the retinoids were analyzed as in (A). Control incubations in the absence of membranes showed that substantially larger amounts of all-*trans*-retinol formed (approximately 10% of the total radioactivity at end of incubation) than when membranes were added. Control values were not subtracted from the indicated values. Individual experiments were run in duplicate or triplicate and repeated several times with the same results. Symbols for (A) and (B): ○, retinyl esters; ●, 11-*cis*-retinol; △, 13-*cis*-retinol; and ▲, all-*trans*-retinol. Error bars indicate SEM with $n$ = 3 to 4.




**Scheme 2.** A coupled reaction could drive the isomerization. Abbreviations: $EB^-$, enzyme active–site nucleophile; and $\Delta G°$, Gibbs free energy.

(*8*). This result is only understandable if C–O bond cleavage occurs during isomerization.

We next attempted to determine the nature of the putative ester substrate of the isomerase. Previously we had shown that when all-*trans*-retinol was added to pigment epithelium membranes it was rapidly converted into retinyl esters, mainly the palmitate ester, before any isomerization was observed (*3, 4*). However, at least in the amphibian case, a small amount of all-*trans*-retinol and all-*trans*-retinal remained as well (*4*). We reinvestigated the processing of all-*trans*-retinol by bovine membranes with some alterations from previous techniques. The membranes were first irradiated with UV light and washed to remove the endogenous retinoids and nicotinamide cofactors. Under these conditions a true tracer experiment can be performed. The addition of [11,12-$^3$H]all-*trans*-retinol to the membranes led to the virtually complete conversion of this material into all-*trans*-retinyl esters (Fig. 2A). Approximately 3% of the all-*trans*-retinol remained, even though an extraction technique was used that overestimates the amount of retinols with respect to retinyl esters (*3*). The percentage of retinyl esters eventually decreased, with a concomitant increase in 11-*cis*-retinol (Fig. 2A). The percentage of all-*trans*-retinol also increased with time, but this was probably due to back isomerization from its 11-*cis*-congener. When the membranes were treated with [11,12-$^3$H]all-*trans*-retinyl palmitate, processing to 11-*cis*-retinol occurred (Fig. 2B). In separate experiments it was shown that [11,12-$^3$H]all-*trans*-retinyl oleate could be converted into 11-*cis*-retinol but not 11-*cis*-retinyl oleate. This is further evidence for the lack of a direct ester-ester isomerization route. These results suggest that under normal physiological conditions a retinyl ester is directly isomerized to 11-*cis*-retinol. Other experiments are consistent with this view. For example, we have not been able to dissociate retinyl ester synthetase activity from isomerase activity during purification of the detergent solubilized materials (*7*), and different reagents, such as ethanol, *p*-hydroxymercuribenzoate, and hydroxylamine, affect both activities in a parallel fashion (*11*). Finally, structure-activity studies are also consistent with the proposed mechanism (*12*).

Our results show that the biological formation of 11-*cis*-retinol requires the input of metabolic energy and that the energy source for this conversion is membrane-derived. A novel group transfer mechanism has been postulated that uses the metabolic energy of an ester moiety of the phospholipid to drive this process. The proposal that membranes may serve as an energy source reveals a previously unsuspected biological function of membranes.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# A G Protein Gamma Subunit Shares Homology with ras Proteins


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**Guanine nucleotide binding proteins (G proteins) that transduce signals from cell surface receptors to effector molecules are made up of three subunits, α, β, and γ. A complementary DNA clone that encodes a 71–amino acid protein was isolated from bovine brain; this protein contains peptide sequences that were derived from the purified γ subunit of $G_i$ and $G_o$. The primary sequence of this G protein γ subunit (Gγ) has 55 percent homology to the γ subunit of transducin (Tγ) and also has homology to functional domains of mammalian ras proteins. The probe for isolating the clone was generated with the use of the polymerase chain reaction (PCR). The extent of divergence between Tγ and Gγ, the isolation of homologous PCR-generated fragments, and the differences between the predicted amino acid sequence of Gγ and that derived from the γ subunit of $G_i$ and $G_o$ indicate that γ subunits are encoded by a family of genes.**


G PROTEINS ARE GENERALLY FOUND associated with cell membranes and are activated by a class of cell surface receptors that includes the β-adrenergic receptor, rhodopsin, and the muscarinic acetylcholine receptor. The activated G protein transduces signals from these receptors to effectors such as phospholipases, guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase, adenylyl cyclase, and ion-conducting channels (*1*). In well-characterized systems such as the G protein activation of cGMP phosphodiesterase and adenylyl cyclase, the G protein α subunit in the activated state binds guanosine 5′-triphosphate, dissociates from βγ, and directly modulates effector function (*2*). On the basis of these


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