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A Model Study of Rhodopsin Regeneration in Visual Cell. Photoisomerization of all-trans Retinal in Phosphatidylcholine Vesicles

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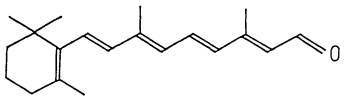
The photochemical behavior has been investigated for all-trans retinal in egg york phosphatidylcholine(EPC) vesicles and dipalmitoylphosphatidylcholine (DPPC) vesicles. In EPC vesicles, 10% of all-trans isomer is isomerized to 11-cis one upon direct irradiation of 15 W-fluorescence light for 180 min. In organic solvents the photoisomerization of all-trans isomer is induced depending on the dielectric constant of solvent. The distributions of retinal isomers in EPC vesicles is similar to those obtained in less polar solvents, such as diethyl ether. Significant difference is not recognized between the distributions of retinal isomers in DPPC vesicles at the above and below T_c. These results indicate that photoisomerization of retinal in PC vesicles is dominated by the electric properties of enviroment of retinal rather than the packing effect of PC membrane.

The vertebrate rhodopsin is embedded in the disk membrane of the rod outer segment (ROS) of a photoreceptor cell. Rhodopsin consists of an apoprotein opsin and a chromophore 11-cis retinal bound to opsin via protonated Schiff base linkage with a side chain of a lysine residue (e.g. Lys^{296} in bovine rhodopsin[1]). The illumination of rhodopsin leads to the isomerization of the 11-cis to all-trans form and finally to the release of the all-trans isomer from opsin.[2-5] Owing to poor solubility of retinal to water the released retinal possibly remains in the disk membrane, which is mainly composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Since PE has an amino group, retinal can form Schiff base with PE in the disk membrane. It has been reported that in the final stage of rhodopsin bleaching (metarhodopsin III) the populations of free, lipid-bound and protein-bound retinal are 34, 15, and 51%, respectively.[6] Although the metabolic pathway of the released retinal remains unclear, a possible one is that it is utilized for the rhodopsin regeneration. In order for rhodopsin regeneration to proceed all-trans retinal should be reisomerized to 11-cis form. When rhodopsin is partially bleached, photochemical reisomerization of the released retinal occurs in the disk membrane.[7] A mechanism for the above process has been proposed by Shichi and Somers[8,9] that the released all-trans retinal is reisomerized photochemically to 11-cis form as protonated retinal-PE Schiff base. This mechasnism is based on the following experimental data: (a) the bleached ROS complex shows an absorption maximum at 458 nm that is similar to the absorption maximum of protonated retinal-PE Schiff base, (b) 450—460 nm light covering the absorption maximum of 458 nm is the most effective on the formation of rhodopsin and (c) the formation of rhodopsin is markedly reduced by partial delipidation and restored by the addition of PE. According to an another report[7] the reisomerization probably occurs photochemically as free retinal, too.

On the other hand it has been reported that retinal-incorporated egg york PC vesicles are damaged by the singlet oxygen produced by the light absorption of retianl.[10-12] In acetonitrile retinal is also damaged by singlet oxygen.[13] Hence, in the disk membrane retinal may be damaged together with membrane by photochemically produced singlet oxygen.

In order to clarify the mechanisms of these reactions in which the released all-trans retinal participates, the photochemical behavior must be investigated for retinal in membrane. Many studies have been reported on photochemical isomerization of all-trans retinal in organic solvents using HPLC (high-performance liquid chromatography) the formation of 11-cis isomer increases with an increases of the polarity of solvents, and the formation of 11-cis isomer increases with an increase of the polarity of solvents, and the formation of 11-cis isomer seems to be reduced by the viscosity of solvents. The data obtained in solution are insufficient to understand the photochemical behavior in the disk membrane since membrane has some characteristics different from solvents i.e., (a) the microscopic inhomogeneity in electric properties arising from the water-hydrocarbon interface and (b) the packing effect of alkyl chains of phospholipids. There have been some reports on the photoisomerization of retinal in organized systems such as micells and membrane.[15,16] However, these reports disconcerned the regeneration process of rhodopsin.

In this report the experiments are designed in order to extract and clarify the effect of the above two factors, namely the inhomogeneity in environment and the packing effect on the photoisomerization of



all-trans retinal

retinal. Sonicated PC vesicle is used as a model of the disk membrane, and all-trans retinal is photoisomerized in it. We shall indicate that the significant amount of 11-cis retinal is photochemically produced in the model membrane system.

Experimental

Materials. Vitamin A was purchased from Riken Vitamin Co. Ltd. All-trans retinal was crystallized by oxidation of vitamin A with manganese dioxide in petroleum ether. Egg york phosphatidylcholine (EPC) and dipalmitoyl phosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. All solvents used in the experiments were guaranteed grade.

Preparation of Vesicles. Chloroform solution of retinal and phospholipids, EPC, or DPPC were evaporated, giving a dry thin film. Molar ratio of retinal and PC was 1 : 5. Phospholipid dispersions were prepared by the addition of 50 mM KCl aqueous solution (1 M=1 mol dm^{-3}) to the dry film and subsequent shaking. The dispersions were then sonicated for 20 min under cooling by ice/water with Ultrasonic Disruptor Model UR-200P (Tomy Seiko Co., Ltd., frequency 20 kHz, output power 200 W). The resultant vesicle solutions were centrifuged at 15000 rpm for 40 min to remove multilayer vesicles and metallic titanium fragments from sonicator's microtip.

Photoisomerization of Retinal in Phospholipids. Irradiation was carried out under 15 W-fluorescence light for an appropriate duration. A 15 W-fluorescence lamp is used as a light source, due to relatively close distribution of wave length compared with that of sun light. 5 cm^3 methanol and 5 cm^3 1,2-dichloroethane were added to the sample and the suspensions were centrifuged at 10000 rpm for 2 min. The lower layer was collected and the organic solvent was removed by evaporation, followed by dissolving to solvent for HPLC. The samples were filtrated through the membrane milipore filters. The HPLC analysis were performed using Waters Model-590 HPLC, with a μ-porasil column (3.9 mm×30cm) under the conditions of flow rate 2.0 cm^3 min^{-1}, 6% diethyl ether/hexane (v/v) as a eluent, and 365 nm UV detection. The eluent and samples used were filtrated through membrane milipore filters before use.

The Photoisomerization of Retinal in Organic Solvents. Retinal was dissolved into the organic solvent used until the absorbance of the solution reached about 2. Irradiation is carried out under 15 W-fluorescence light for 90 min. After irradiation the solvents were removed by evaporation, followed by redissolved to solvent for HPLC. The HPLC analyses were similarly performed to the above procedure.

DSC Experiments. Retinal incorporated vesicles were prepared as mentioned above. Sample was injected into a small volume pan and the characteristics were recorded on DSC 10 (Seiko I&E) with heating rate of 0.5 °C min^{-1}.

All manipulations were performed under red dim light except the photoisomerization procedures.

Results

Estimation of Retinal Photo-Damage in EPC Vesicles. In Fig. 1 is shown the HPLC chromatogram of extract from retinal/EPC vesicles irradiated for 40 h. The peakes appearing in the retention time range of 4 to 10 min correspond to configurational isomers of retinal. Additional three broad peakes were found in the retention time range of 15 to 40 min. One of the three peaks found just before 20 min increases with the duration of irradiation. The identification of this peak has not been performed in particular. The hemiacetal isomer of 5,8-peroxyretinal is one of possible candidates, because 5,8-peroxyretinal is produced by the photoisomerization of all-trans retinal in aerated solution,[13] and easily isomerized to the hemiacetal isomer.[17] An absorption coefficient was reported for hemiacetal isomer of 5,8-peroxyretinal ($\varepsilon_{320\ nm}$=33000 in hexane[17]). This value was used in order to estimate the extent of oxidation.[16] According to the estimation, only 3% of retinal is damaged (converted to other compounds) during irradiation for 3 h. During irradiation for 40 h the damaging of retinal increased up to 13%.

Determination of Optimum Irradiation Time. In Fig. 2 the fractional populations of retinal isomers were plotted against a function of photo-irradiation

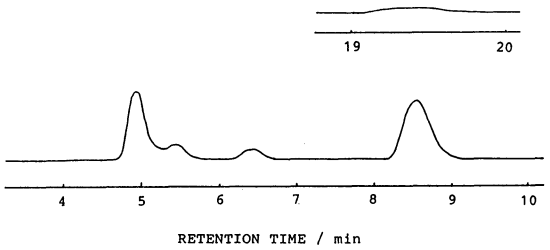


Fig. 1. HPLC chromatogram of extract from retinal/EPC vesicles after irradiated for 40 h.

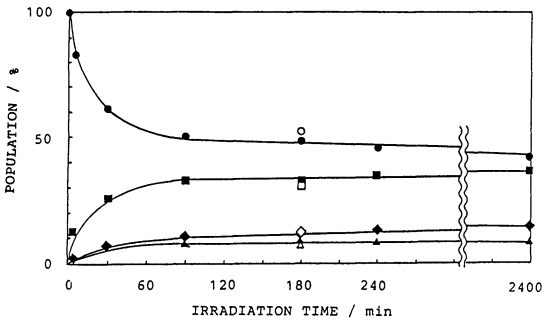


Fig. 2. The plots of the populations of retinal isomers formed in EPC vesicles vs. the duration of irradiation (at room temperature); ●○ all-trans, ■□ 13-cis, ◆◇ 11-cis, ▲△ 9-cis. The solid and open simbols correspond to the composition obtained in the absence and the presence of α-tocopherol, respectively. [retinal]:[EPC]=1 : 5.

Table 1. Populations of Retinal Isomers Formed in EPC Vesicles

Isomerization conditions	Isomer formed/%				
	13-cis	11-cis	9-cis	7-cis	All-trans
Irradiation	32.6	10.2	9.0	Trace	48.3
Dark incubation	2.4	Trace	Trace	Trace	97.6

Table 2. Populations of Retinal Isomers Formed by Irradiation in Organic Solvents

Organic solvent	Dielectric constant	Isomer formed/%					Distribution type[a)]
		13-cis	11-cis	9-cis	7-cis	All-trans	
Hexane	1.88	25.7	Trace	3.4	Trace	70.9	A
Diethyl ether	4.34	37.3	6.9	6.2	Trace	49.5	B
Chloroform	4.81	29.0	28.2	10.3	1.1	31.4	B
Ethyl acetate	6.02	34.5	14.9	12.4	Trace	38.2	B
Methanol	32.70	14.7	35.7	11.5	Trace	38.1	C
Acetonitrile[b)]	37.50	18.7	43.4	11.7	5.8	20.4	C

a) See text. b) Ref. 12.

time. Fig. 2 indicates that the populations of retinal isomers approaches gradually to those at 40 h. In order to minimize the retinal damaging and to approach populations close to those in the photoequilibrium state, we determined the best irradiation time to be 180 min.

α-Tocopherol is well-known as a scavenger of singlet oxygen in biomembranes.[18-20] Retinal was photoisomerized in α-tocopherol/EPC vesicles. In Fig. 2 are shown the results for 180 min irradiation. The similarity of these two distributions of isomers obtained in the absence and in the presence of α-tocopherol indicates that the oxidation of retinal inaffects the isomerization behavior of retinyl polyene during irradiation for 180 min.

Photoisomerization of Retinal in EPC Vesicles. In Table 1 are summerized the populations of retinal isomers formed during direct irradiation (upper) and dark incubation (lower) in EPC vesicles. The isomerization time is 180 min in each case. Upon irradiation for 180 min in EPC vesicles 13-cis, 11-cis, and 9-cis isomers are formed with the populations of 33, 10, and 9%, respectively. 11-cis Isomer (important in rhodopsin regeneration) is formed less than 13-cis isomer. On the other hand, upon dark incubation for 180 min 11-cis and 9-cis isomers are not formed but only 13-cis isomer is formed with the population of 2.4%. No formation of 11-cis isomer is consistent with an earlier report.[21] It is noted that only under the irradiation conditions, 11-cis isomer can be formed.

Photoisomerization of Retinal in Organic Solvents. In Table 2 are summerized the populations of retinal isomers in the photostationary state in various organic solvents. By monitoring the change of the UV absorbance, it is confirmed that the photostationary state is attained by irradiation of 90 min. In order to investigate the dependence of isomer populations on the polarity of solvent, various types of solvents were selected so as to cover the wide range of the dielectric constants. In hexane 13-cis and 9-cis isomers were formed with the populations of 26 and 3%, repectively. 11-cis Isomer was not formed upon irradiation in hexane. In the solvents with relatively low dielectric constants, 4 to 6, 11-cis isomer is less formed than 13-cis isomer. In diehtyl ether, for example, 13-cis, 11-cis, and 9-cis isomers are formed with the populations of 37, 7, and 6%, respectively. In the solvents with relatively high dielectric constants, 30 to 40, 11-cis isomer is more formed than other cis isomers. In methanol, for example, 13-cis, 11-cis, and 9-cis isomers are formed with the populations of 15, 36, and 12%, respectively. On the basis of the relative population of 11-cis isomer against other cis isomers, we can classify the isomer distributions in those solvents into three types, named type A, type B, and type C (see the last column of Table 2). Type A is characterized as no formation of 11-cis isomer. Type B is characterized as the cis isomer formtions in the order of 13-cis>11-cis>9-cis. And type C is characterized as the preferencial formation of 11-cis isomer among cis ones. According to this classification, the formation of 11-cis isomer increases in the order of type A<type B<type C. We, therefore, found a good correlation between the distributions and the polarity (dielectric constant) of the solvents. That is to say, the relative

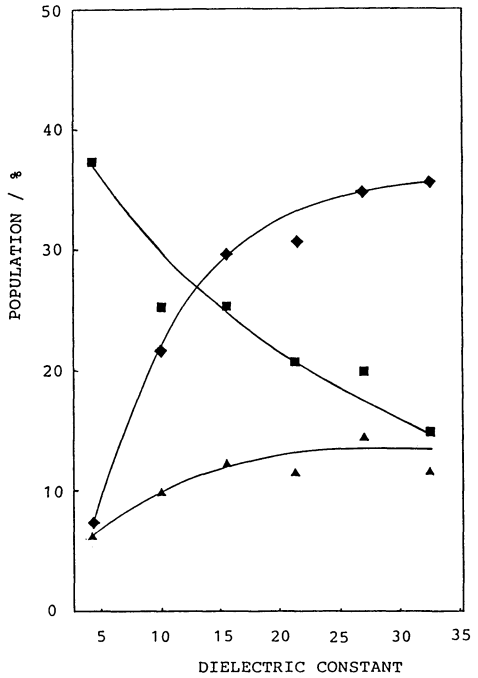


Fig. 3. The dielectric-constant dependence of the populations of retinal isomers formed in diethyl ether/methanol mixture (at room temperature); ■ 13-cis, ◆ 11-cis, ▲ 9-cis.

population of 11-cis retianl increases with an increase of the polarity of the environments.

Photoisomerization of Retinal in Diethyl Ether-Methanol Mixture. In order to confirm the polarity dependence of distributions of cis isomers, we used diethyl ether-methanol mixtures as the solvents for the isomerization reaction. In Fig. 3 the populations of cis isomers are plotted against the dielectric constants of solvents. The dielectric constans are calculated on the basis of linear combination of those of two components. In Fig. 3 is shown that with an increase in the dielectric constant the population of the 11-cis isomer increases while that of 13-cis isomer decreases. Irrespective of solvent composition, the 9-cis isomer is smaller than both 11-cis and 13-cis isomers. At low dielectric constants, 4 to 10, the population of 11-cis isomer is smaller than that of 13-cis isomer, and thus the isomer distributions is classified into the type B. At relatively higher dielectric constants, 15 to 33, the order of the formation of 11-cis and 13-cis isomers is altered and the isomer distributions is classified into the type C. These findings are confirmations for the correlation between the polarity of solvent and the distributions of retianl isomers.

We attempt to apply the above classification to the isomer distributions resulting from the photoisomerization of retinal in EPC vesicles (Table 1, upper). The isomer distribution in EPC vesicles can be assigned to one of these three types defined the above, namely, type B. Since the isomer distribution in EPC vesicles is comparable to those obtained in solutions of less polar solvents, especially in diethyl ether, we can suppose that the photoisomerization of retinal in EPC vesicles is also dominated by the polarity of its surrounding environment, and that retinal receives photon in an apolar region, such as hydrocarbon core of the vesicles.

Photoisomerization of Retinal in DPPC Vesicles. The gel-liquid crystal phase transition temperature T_c was measured for retinal-containing DPPC vesicles. From DSC (differencial scanning calorimetry) chromatogram, T_c of retinal/DPPC vesicles (molar ratio 1 : 5) was determined to be 37.7 °C. The T_c obtained here is lower by ca. 4.5 °C than that of pure DPPC vesicles, 41.2 °C. The lowering of T_c induced by incorporation of retinal is consistent with a previous report.[22]

In Table 3 are summerized the populations of retinal isomers formed during irradiation for 180 min in DPPC vesicles. In order to investigate the effect of mobility of lipids on isomerization process, irradiation were carried out at the temperatures above and below T_c, 51 °C and 24 °C, respectively. In the liquid crystal state (above T_c), 13-cis, 11-cis, and 9-cis isomers are formed with the populations of 36, 5, and 10%, respectively. In the gel state (below T_c), 13-cis, 11-cis, and 9-cis isomers are formed with the populations of 32, 5, and 10%, respectively. Both distributions at these two temperatures correspond to the medium type between A and B. No significant difference is recognized between the distributions formed at these two different temperatures. These findings indicate that the photoisomerization of retinal is inaffected by the change in the packing effect depending on the state of alkyl chain.

Table 3. Populations of Retinal Isomers Formed by Irradiation in DPPC Vesicles

Temperature /°C	Isomer formed/%				
	13-cis	11-cis	9-cis	7-cis	All-trans
51 (above T_c)	36.4	4.6	10.2	Trace	48.8
24 (below T_c)	32.4	5.3	11.9	Trace	50.3

Discussion

In the final stage of the rhodopsin bleaching, the major fraction of the retinal molecules released from opsin dissociates into free form in the disk membrane.[6] Thus, if these retinals are isomerized to 11-cis form and subsequently bind to opsin, the significant amount of rhodopsin is expected to be regenerated. It has been reported that second step (binding to opsin) in the regeneration process proceeds efficiently in the disk membrane.[9] The efficiency of rhodopsin regeneration, therefore, depends on that of the first step (isomerization to 11-cis isomer), which can be estimated from the present data. Irradiation experiments were carried out using the usual light source (15 W-fluorescence light), which does not contain the light of ultraviolet region (<400 nm). The present results indicate that under the irradiation of visible rays all-trans isomer (λ_{max}=387 nm) is efficiently isomerized to cis isomers in lipid membrane. Consequently, 10% of the all-trans is isomerized to 11-cis and simultaneously the comparable amount of 9-cis is produced. 9-cis Isomer can also bind with opsin, giving isorhodopsin. On the other hand, during dark incubation, 9- and 11-cis isomers are not produced in the lipid membrane. These results suggest that the significant amount of natural pigments can be regenerated through the light adaption of the disk membrane containing the bleached rhodopsin.

The population of 11-cis (10%) obtained here are inconsistent with that (0.3%) appearing elsewhere.[14] The origin of this discrepancy may be attributable to the difference in irradiation intensity. 400 W-mercury lamp was used in Ref.13. Stronger irradiation may induce various side reactions involving the formation of epoxyretinal.

The isomer distribution in EPC vesicles is similar to those in the medium of relatively lower dielectric constant. This finding suggests that the photoisomerization behavior of retinal in EPC vesicles is mainly controlled by electric properties of its environment. And the principal moiety of the retinal molecule should be located in a hydrophobic region of

EPC vesicles. In nonpolar solvent such as hexane 11-cis isomer is, however, not formed. Thus in EPC vesicles the retinyl polyene must be partially exposed to the polar region of EPC vesicles. Probably the -C=O group of retinal is in contact with the polar head groups of lipids. Such a location and orientation of retinal are consistent with the results of ESR[23] and NMR[15,24] studies on retinal-vesicles and -micelles systems. However, on the basis of the present results, the inhomogeneity of polarity in EPC vesicles is not necessarily important for the control of the photoisomerization pathway of retinal. Rather, the idea of the average polarity of the environment is sufficient to explain the observed isomer distribution.

No significant difference appearing in the isomer distributions in DPPC vesicles at above and below T_c. This finding suggests directly that the change in the packing effect induced by the phase transition of lipid inaffects the photoisomerization behavior of retinal. This is consistent with the fact that the trend of the isomer distribution is not largely different from that observed in solution. The incorporation of retinal into vesicles causes to increase lipid-lipid distance, leading to the decrease in the cooperativity of lipid molecules. This is supported by the facts that the incorporation of retinal leads to increasing the proton permiation,[25] broadening of the DSC transition[22] and lowering of the Tc of DPPC vesicles.[22] The weakening of the packing facilitates the isomerization of retinal, which may inaffects in lipid packing.

The isomer distribution attained in EPC vesicles is compared with that in DPPC vesicles. The formation of 11-cis in DPPC vesicles are smaller than in EPC vesicles, and the formation of 9-cis and 13-cis isomers in DPPC are larger than those in EPC vesicles. As indicated in Fig. 3 in the medium of lower dielectric constant (ca. 10), the populations of these isomers dramatically changes in the region of dielectric constant. In other words, the slight variation of the environment causes the largely different isomer populations in a lower dielectric medium. EPC and DPPC are different in the spiecies of fatty acid chains, indicating variation of the dielectric properties of hydrophobic core of membrane, and in the extent of retinal penetration. This is the main origin of the observed difference in the isomer distribution between EPC and DPPC vesicles.

In summary, we used the PC vesicles as a model of disk membrane. In EPC vesicles 10% of 11-cis retinal are formed by irradiation of 15 W-fluorescence light. Since 11-cis isomer is not formed during dark incubation, photoisomerization process is effective to form 11-cis isomer. On the other hand, the photoisomerization data in organic solvents are useful to explain the photoisomerization in EPC vesicles, and to estimate the environment of retinal in PC vesicles.

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