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Activation of Phosphodiesterase by Rhodopsin and its Analogues*

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Abstract. Activation of guanosine 3′,5′-cyclic monophosphate (cGMP) phosphodiesterase (EC 3.1.4.35.) in frog rod outer segment membrane by rhodopsin and its analogues was investigated. The Schiff-base linkage between opsin and retinal in rhodopsin was not always necessary for the phosphodiesterase activation. The binding of β-ionone ring of retinal to a hydrophobic region of opsin was not enough to induce the enzyme activation. A striking photo-activation of the enzyme was induced by photo-isomerization of rhodopsin analogues from *cis* to *trans* form. It seems probable that an "expanded" conformation of opsin around the retinylidene chromophore induced by the *cis* to *trans* isomerization may be the trigger for the activation of phosphodiesterase. On the other hand, the phosphodiesterase in frog rod outer segment was activated by warming of bathorhodopsin to −12° C and then incubating it at the same temperature. Thus, metarhodopsin II or an earlier intermediate than metarhodopsin II should be a direct intermediate for the enzyme activation.

Key words: Cyclic GMP – Phosphodiesterase – Rhodopsin – Iodopsin – Retinal analogues – Rod outer segment

Introduction

When a rhodopsin molecule absorbs a photon, it bleaches over a series of intermediates to be photolyzed into all-*trans*-retinal (chromophore) plus opsin (apoprotein). The photolysis process has intensively been studied by low temperature spectrophotometry (Yoshizawa and Shichida 1982). On the other hand, biochemical studies showed that guanosine 3′,5′-cyclic monophosphate (cGMP) phosphodiesterase in dark-adapted frog rod outer segments was

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activated by light in the presence of nucleoside triphosphate (Miki et al. 1973). Since cGMP phosphodiesterase in vertebrate rod cell can be activated by photolysis of rhodopsin in the presence of both GTP and GTP-binding protein, it has been proposed that cGMP may play a role of an intracellular transmitter in the visual transduction process (Hubbell and Bownds 1979; Pober and Bitensky 1979). Now the question arises which intermediate of bleaching of rhodopsin can activate the phosphodiesterase and by what mechanism it can trigger the enzyme activation. In order to solve this question, we examined the enzyme activations both by various kinds of retinal and opsin analogues and by a low temperature technique.

Materials and Methods

All manipulations except irradiation of samples were performed under infrared light (> 800 nm) using darkroom goggles (Type 5156, N. V. Optische Industrie, Delft, The Netherlands).

Preparation of Frog Rod Outer Segment

Frog rod outer segment membrane was prepared from retinas of 12 h dark-adapted frog (*Rana catesbeiana*) by 43% (w/w) sucrose flotation method (Fukada et al. 1981) and suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$ and 2 mM dithiothreitol (dark-adapted frog rod outer segment membrane). This preparation contains cGMP phosphodiesterase with components necessary for photo-activation of the enzyme in the presence of GTP, so that it was used as a source of the enzyme within the day prepared.

The preparation was further purified by a sucrose density gradient centrifugation, followed by an EDTA washing for removing the phosphodiesterase (Fukada et al. 1981). The phosphodiesterase-depleted rod outer segment membrane thus obtained was irradiated in the presence of neutralized hydroxylamine (100 mM) for converting rhodopsin into retinal-oxime plus opsin and then extracted the retinal-oxime with light petroleum (Fukada and Yoshizawa 1981). We call this preparation "opsin membrane" which was used as an opsin source.

Preparation of Bovine Opsin

Bovine rod outer segment membrane was purified by almost the same method as the preparation of frog opsin membrane, except that the first sucrose flotation step was carried out using 34% (w/w) sucrose buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; ph 7.0). The opsin membrane extracted retinal-oxime with light petroleum was solubilized in 2% digitonin buffered with 10 mM HEPES (pH 7.0), and then spun down to get a clear supernatant (bovine opsin).

Preparation of Chicken Outer Segment

Freshly decapitated chicken heads were dark-adapted for 6 h, from which the eyeballs were enucleated. Chicken outer segment membrane was prepared from the retinas by 40% (w/w) sucrose flotation method (Fukada and Yoshizawa, 1982) and then suspended in an isotonic solution (20 mM Tris-HCl, 120 mM NaCl, 3 mM $MgCl_2$ and 2 mM dithiothreitol, pH 7.8). Further purification was carried out by a sucrose density gradient centrifugation (Fukada and Yoshizawa 1982), followed by an EDTA washing for removing phosphodiesterase. The purified outer segment membrane thus obtained was completely bleached by irradiation in the presence of neutralized hydroxylamine (50 mM) to get a mixture of photopsin (protein moiety of iodopsin) and scotopsin (protein moiety of rhodopsin), followed by washing with light petroleum for extracting the retinal-oxime. After centrifuging it, the precipitate was suspended in 18% (w/w) sucrose buffered with the isotonic solution, and then sonicated for 3 min. The preparation was spun down to remove some black membraneous materials. The final supernatant was used as a chicken opsin vesicle preparation.

Preparation of Rhodopsin Analogues

Most retinal analogues were synthesized in Professor K. Tsukida's laboratory. They were purified by means of high-performance liquid chromatography just before use and dissolved in ethanol. Each of the retinal analogues was mixed with frog opsin membrane in a 1 : 1 molar ratio and then incubated in the dark at 30° C for 1−12 h to form its rhodopsin analogue.

Free retinal analogues which were used as samples were dispersed in 10 mM Tris-HCl buffer containing 1 mM $MgCl_2$ (pH 8.0).

Chicken iodopsin was regenerated from 11-*cis*-retinal and chicken opsin vesicle containing photopsin and scotopsin. If a little less 11-*cis*-retinal (80% in molar ratio of iodopsin content in the sample) than would saturate the photopsin is added to the chicken opsin vesicle and incubated at 10° C for 30 min, only iodopsin regenerates virtually (Matsumoto and Yoshizawa 1982), because the rate of regeneration of iodopsin from photopsin and 11-*cis*-retinal is about 500 times faster than that of rhodopsin from scotopsin and 11-*cis*-retinal (Wald et al. 1955). The sample thus prepared contained 72% of the original iodopsin and almost no rhodopsin, which was confirmed by spectrophotometry.

Assays

Phosphodiesterase activity was estimated from the amount of 5′-GMP produced in a reaction mixture (20 µl) composed of 2 mM [^{3}H]cGMP (1.5 Ci/mol), 10 µM GTP, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM Tris-HCl (ph 8.0), and 15−40 µg membrane protein. The 5′-GMP was isolated from 3′,5′-cGMP by inorganic salt co-precipitation (Chan and Lin 1974) in the experiment of Table 1

or by thin-layer chromatography on polyethyleneimine cellulose (Keirns et al. 1974) in the experiment of Fig. 1.

Concentrations of rhodopsin, rhodopsin analogues and iodopsin were estimated from their absorbances at λ_{max}s using their molar extinction coefficients.

The concentration of protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results and Discussion

1. Rhodopsin and its Isomers

As shown in Table 1, frog opsin membrane (I) had no ability for activation of the phosphodiesterase in the dark-adapted frog rod outer segment membrane. Addition of 11-*cis*-, 9-*cis*- or 7-*cis*-retinal (II, III, or IV), each of which has no activation ability, to the opsin membrane yielded 11-*cis*-, 9-*cis*- or 7-*cis*-rhodopsin (II, III, or IV). They also displayed no activation ability. When they are irradiated by visible light, remarkable activation abilities become manifest. Since these rhodopsin isomers yield the same bathorhodopsin (Yoshizawa and Wald 1963; Kawamura et al. 1980), they should show similar activation abilities. However, slightly different activation abilities among these isomers seem to be due to differences in photosensitivity of each isomer for the irradiation light and its intensity. 9,13-*dicis*-rhodopsin also displayed a similar activation ability in the light (Ebrey et al. 1980). These results are in fairly good agreement with electrophysiological experiments in isolated skate retinas; the isomer which can activate the phosphodiesterase when irradiated, bings about a significant decrease in threshold of the photoreceptor potential (Crouch and Pepperberg 1978; Pepperberg et al. 1978).

2. Modification at the Schiff-Base Linkage

In the course of preparation of opsin, we found that the opsin having a retinal-oxime (V) showed some ability for activation of the phosphodiesterase. This result indicates that the Schiff-base linkage between opsin and retinal is not always necessary for the enzyme activation. However, it is not clear yet whether or not the retinal-oxime still remains at the retinal binding site. Anyway, addition of 11-*cis*-retinal to the opsin having a retinal-oxime yielded 11-*cis*-rhodopsin which had no activation ability (data not shown).

Next we examined the enzyme activation ability of N-retinyl-opsin, the reduced chromophore of which still remains attached at the retinal binding site of opsin. N-retinyl-opsin was prepared at 0° C by irradiation (> 560 nm) of phosphodiesterase-depleted frog rod outer segment membrane in the presence of 100 mM $NaBH_4$ (Bownds and Wald 1965). The excess $NaBH_4$ was removed by dialysis. As shown in line VI, N-retinyl-opsin did activate the phosphodiesterase. This activation ability was not due to a non-specific effect of $NaBH_4$ on the opsin other than its chromophore, because the similarly $NaBH_4$-treated

membrane *without irradiation* induced no enzyme activation (Fukada et al. 1982) (The Schiff-base linkage is only attacked when irradiated).

The results obtained above indicate that, though the modification of the Schiff-base linkage reduces the activation ability to about one third of that of bleached product of rhodopsin, the phosphodiesterase activation by an intermediate of bleaching of rhodopsin is not always due to the exposed N-retinylidene Schiff-base.

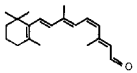
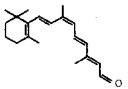
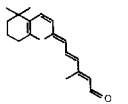
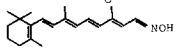
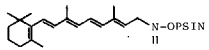
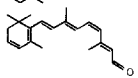
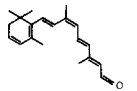
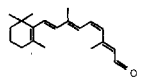
3. *Retinal$_2$-Pigment and Chicken Iodopsin*

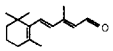
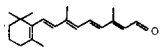
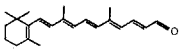
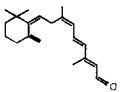
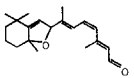
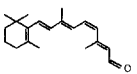
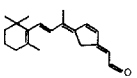
Visual pigments of fresh water fishes and amphibia have a 3-dehydro-retinal (retinal$_2$) as their chromophore (Wald 1960; Reuter et al. 1971). However, no attempt was made to measure the phosphodiesterase activation by porphyropsin or cyanopsin. Here we have prepared 11-*cis*- and 9-*cis*-3-dehydro-rhodopsins from chemically synthesized 11-*cis*- and 9-*cis*-3-dehydro-retinal (VII and VIII, respectively) and frog opsin (protein moiety of rhodopsin, not of porphyropsin). These artificial pigments induced remarkable activation of phophodiesterase when irradiated, which are quite similar to activations due to the rhodopsin isomers having retinal$_1$. This indicates the cyclohexadiene ring can be substituted for the β-ionone ring to preserve the ability for enzyme activation. Thus, naturally occurring retinal$_2$-based pigments may be expected to activate phophodiesterase when irradiated, though we have no information about the difference in apoprotein.

Next, the apoprotein was changed from frog opsin to chicken cone opsin (photopsin). 11-*cis*-retinal binds with the photopsin to yield iodopsin (Wald et al. 1955). At present, attempts to isolate a membrane (suspension or vesicle) containing iodopsin (or photopsin) without any rhodopsin (or scotopsin) from chicken retina have remained without success. So we used mixed membrane containing photopsin and scotopsin from which a mixture of iodopsin and scotopsin (and no rhodopsin, see Materials and Methods) in the membrane was prepared. Conveniently, scotopsin has no activation ability on frog phosphodiesterase, so that one can virtually neglect the existence of scotopsin. As shown in line IX, chicken photopsin has no activation ability on the frog enzyme. The regenerated iodopsin, however, induced a remarkable activation of the enzyme without irradiation. By irradiation it was enhanced about twice (X), which was quite different from the activation by rhodopsin. This strange observation may be related to the different behavior of iodopsin from that of rhodopsin. For example, the regeneration of iodopsin is about 500 times faster than that of rhodopsin (Wald et al. 1955) and the chromophore of iodopsin is easily attacked by hydroxylamine (Wald et al. 1955), $NaHB_4$ or 9-*cis*-retinal (Matsumoto et al. 1975), while these reagents have no effect on that of rhodopsin. These features seem to reflect a fluctuating conformation of iodopsin around the chromophore, bearing some resemblance to an "expanded" conformation (described below), which may trigger the enzyme activation in the dark.

Though we have observed that the chicken cone and/or rod outer segment(s) contain(s) phosphodiesterase to be activated in the presence of GTP, we cannot

Table 1. Activation of phosphodiesterase in frog rod outer segment membrane by rhodopsin analogues. Each rhodopsin analogue (10 µl) was mixed with the dark-adapted frog rod outer segment membrane (100–400 µl) containing 15–20 µM rhodopsin, and then the phosphodiesterase activity was measured in the dark. The final concentration of each rhodopsin analogue in the mixture was 1 µM. In the cases XII–XV, opsin concentration was adjusted to 1 µM. The 'phosphodiesterase activation by mixing (%)' was calculated by (R–D)/(L–D), where R, D, and L are phosphodiesterase activities of the sample containing a retinal analogue or a rhodopsin analogue with frog rod outer segment membrane, the dark-adapted frog rod outer segment membrane and the light-adapted (> 560 nm at 0° C for 1 min) frog rod outer segment membrane. The values were expressed as a mean ± SD in four to 18 experiments. One hundred percent corresponds to 0.81 ± 0.12 µmol cGMP hydrolyzed per min per mg protein and the ratio D/L was 10–22%.

	Rhodopsin analogue			Absorption maximun [nm]	Phosphodiesterase activation by mixing [%]
	Retinal analogue		Opsin		
I			FROG OPSIN		0 ± 1
II		11-*cis*-retinal		376[a]	0 ± 1
			+ OPSIN	502	2 ± 3
			+ OPSIN + irr[c]		52 ± 11
III		9-*cis*-retinal		373[a]	0 ± 2
			+ OPSIN	488	0 ± 1
			+ OPSIN + irr[d]		55 ± 8
IV		7-*cis*-retinal		359[b]	0 ± 3
			+ OPSIN	456	6 ± 3
			+ OPSIN+ irr[e]		44 ± 9
V		retinal-oxime	in OPSIN	367	20 ± 5
VI		N-retinyl-opsin	N-OPSIN	330	17 ± 3
VII		11-*cis*-3-dehydro-retinal		393[a]	0 ± 1
			+ OPSIN	522	0 ± 2
			+ OPSIN + irr[f]		31 ± 7
VIII		9-*cis*-3-dehydro-retinal		391[a]	1 ± 2
			+ OPSIN	504	1 ± 3
			+ OPSIN + irr[c]		34 ± 8
IX			CHICHEN PHOTOPSIN		0 ± 2
X		11-*cis*-retinal		376[a]	0 ± 1
			+ PHOTOPSIN	572	32 ± 5
			+ PHOTOPSIN + irr[g]		65 ± 8

XI			FROG OPSIN		0 ± 1
XII		β-ionone		297[a]	0 ± 1
			+ OPSIN (1:1)		3 ± 3
			+ OPSIN (3:1)		1 ± 2
XIII		β-ionylideneacetaldehyde		280/322[a]	1 ± 2
			+ OPSIN (1:1)		0 ± 2
			+ OPSIN (3:1)		0 ± 1
XIV		all-*trans*-retinal		381[a]	1 ± 2
			+ OPSIN (1:1)		13 ± 2
XV		retinylideneacetaldehyde		393[a]	0 ± 1
			+ OPSIN (1:1)		0 ± 2
			+ OPSIN (3:1)		2 ± 2
XVI		9-*cis*-retro-γ-retinal		338[a]	0 ± 2
			+ OPSIN	420	13 ± 2
			+ OPSIN + irr[h]		12 ± 8
XVII		11-*cis*-5,8-epoxy-retinal		329[a]	1 ± 3
			+ OPSIN	407	3 ± 3
			+ OPSIN + irr[i]		19 ± 6
XVIII			BOVINE OPSIN		2 ± 2
XIX		11-*cis*-retinal		376[a]	0 ± 2
			+ OPSIN	498	7 ± 2
			+ OPSIN + irr[c]		65 ± 8
XX		cyclopentatrienylidene		405[a]	1 ± 3
		11-*cis*-locked-retinal	+ OPSIN	495	6 ± 3
			+ OPSIN + irr[j]		8 ± 2
			+ OPSIN + irr[k]		8 ± 4

[a] Measured in ethanol, [b] Measured in n-heptane, irr: Irradiated at 0° C, [c] > 560 nm (irradiated with light at wavelengths longer than 560 nm), [d] > 530 nm, [e] > 490 nm, [f] > 580 nm, [g] at 670 nm, [h] > 460 nm, [i] > 440 nm, [j] > 560 nm, [k] > 560 nm then at 433 nm

decide yet whether or not the cone outer segment contains photo-activated phosphodiesterase (Fukada and Yoshizawa 1982), because iodopsin has an activation ability on the rod enzyme. In addition, it was reported that light- and dark-adaptation gave no effect on cGMP level in ground squirrel retina composed of cone cell dominantly (De Vries et al. 1979). This suggests that the cone cell might not contain the photo-activated phosphodiesterase and, therefore, cGMP might not act as an intracellular transmitter in its visual transduction process.

According to Ebrey et al. (1980), octopus rhodopsin has an ability for photo-activation of bovine phosphodiesterase similar to bovine rhodopsin, while bacteriorhodopsin has no ability for the enzyme activation both in the dark and light. Anyway, iodopsin is the only natural visual pigment which exhibits the high ability for activation of the enzyme without irradiation, as far as we know.

4. Change in the Length of Retinal Polyene Side-chain

An 11-*cis*-retinal binds with opsin through both a Schiff-base linkage between its aldehyde group and ε-amino group of lysine residue and a hydrophobic bond between its β-ionone ring and hydrophobic region of opsin (β-ionone ring-binding site: Matsumoto and Yoshizawa 1975). β-ionone or β-ionylidene-acetaldehyde forms a complex with bovine opsin through the hydrophobic bond (Matsumoto and Yoshizawa 1975; Daemen 1978; Towner et al. 1981) but not through the Schiff-base linkage, so that the absorption spectrum does not show a strong red shift. Here we have made a comparison of abilities for frog phosphodiesterase activation among the complexes or mixtures composed of frog opsin and β-ionone (XII), β-ionylideneacetaldehyde (C_{15}-retinal: XIII), retinal (XIV) or retinylideneacetaldehyde (C_{22}-retinal: XV) in all-*trans* form. These retinal analogues have the same β-ionone ring and a polyene side-chain different in length. All the enzyme activation abilities of these complexes and mixtures except the case of all-*trans*-retinal showed the zero levels in the dark (XII-XV). Irradiations of these complexes induced no enzyme activation (data not shown). This indicates that any ability for the enzyme activation is not endowed to the opsin by the hydrophobic binding of β-ionone ring to it, but is dependent on the length of the polyene side-chain of the retinal analogues. The fact that all-*trans*-retinal slightly activated the enzyme suggests that the reversal pathway of photolysis of rhodopsin might take place, i. e., all-*trans*-retinal might form a complex with the opsin through not only the hydrophobic bond but also the Schiff-base linkage. The length of the polyene side-chain of retinal in all-*trans* form in retinal binding site of opsin seems to be important for the enzyme activation.

5. Modification at the Conjugate Double-bond of the Retinal Side-chain

The phosphodiesterase activation by retro-γ-rhodopsin (XVI) which was modified at the conjugate double-bond in its chromophore but has almost the

same length of the side-chain was examined. Retro-γ-rhodopsin caused a little phosphodiesterase activation in the dark, but its photo-product at 0° C showed no further enzyme activation. Since the conjugate double-bond system is dissected into diene and trienal chromophoric groups in retro-γ-retinal (compare XVI with III), the single bonds of C_7-C_8 and C_8-C_9 can freely rotate in this analogue. In the case of rhodopsin isomers which can activate phosphodiesterase in the light, the distance between the center of β-ionone ring and the Schiff-base nitrogen (l, longitudinal length) increases about 20% by the isomerization of the 11-*cis* (l = 10.1 Å) or 9-*cis* chromophore (l = 9.78 Å) to the all-*trans* form (l = 12.4 Å) (Matsumoto and Yoshizawa 1978). This may cause some expansion of opsin by increase of the longitudinal length. On the other hand, it seems possible that 9-*cis*-retro-γ-rhodopsin has a 7,9-*dicis* like conformation (180° rotation around the C_7-C_8 single bond; see Fig. 2) in the retinal binding site of opsin. If so, some expansion of opsin around the binding site, judging from the fact that the longitudinal length of 7,9-*dicis* isomer of retinal (l = 11.1 Å: Matsumoto and Yoshizawa 1978) is slightly longer than that of 9-*cis* isomer (l = 9.78 Å), would cause a slight enzyme activation without irradiation. When the retro-γ-rhodopsin was irradiated, the all-*trans* chromophore in its photo-product could not further increase the longitudinal distance due to some folding at the single bonds of C_7-C_8 and C_8-C_9, resulting in no further activation of the enzyme (see Fig. 2).

The idea mentioned above may be supported by an experiment of the enzyme activation by 5,8-epoxy-rhodopsin (XVII). This rhodopsin analogue has a dissected conjugate double-bond system in the chromophore similar to that of retro-γ-rhodopsin, but a rigid cyclic conformation in which C_6-C_7 double bond and C_7-C_8 single bond neither rotate nor fold because C_5 and C_8 are bridged with an oxygen atom, so that photo-activation of phosphodiesterase by this rhodopsin analogue was expected. In fact, 11-*cis*-5,8-epoxy-rhodopsin exhibited no enzyme activation in the dark, and moderate enzyme activation in the light (XVII). Relatively small activation ability in the light, which is about one third of that of irradiated rhodopsin (II), would be due to the difference in the longitudinal length in their all-*trans* chromophores between them. The longitudinal length of all-*trans*-retinal is 12.4 Å, while that of all-*trans*-5,8-epoxy-retinal is 11.4 Å, estimating from CPK molecular model. This may indicate the relatively small enzyme activation ability.

From these results, it seems reasonable to propose that an "expanded" conformation of opsin around the retinylidene chromophore induced by the photo-isomerization of the 11-*cis* chromophore to the all-*trans* form (the first event) should be the second event for the activation of phosphodiesterase.

6. *Prohibition of cis-trans Isomerization*

Two kinds of retinal analogues have been synthesized in order to prohibit the isomerization at $C_{11}-C_{12}$ double bond: One is locked with cycloheptene (Akita et al. 1980) and the other with cyclopentene (Ito et al. 1982). Both analogues bind with bovine opsin to produce rhodopsin analogues having their λ_{max}s at

around 500 nm (Akita et al. 1980; Ito et al. 1982). We have tested cyclopentatrienylidene 11-*cis*-locked-retinal (11-*cis*-locked-retinal: XX) and its pigment for ability to activate the phosphodiesterase. Since this retinal analogue scarcely binds with frog opsin (in membrane or in digitonin extract) or bovine opsin in membrane, a digitonin extract of bovine opsin was used as a starting material. As a control, 11-*cis*-rhodopsin regenerated from it was tested on the ability for activation of phosphodiesterase in the dark-adapted frog rod outer segment membrane. The result obtained was almost similar to that of frog 11-*cis*-rhodopsin (compare XIX with II). A little activation ability of the bovine rhodopsin without irradiation should be ascribed to the effect of digitonin as a solubilizer. Subsequently the 11-*cis*-locked-rhodopsin was tested. This analogue showed a reversible photochemical reaction at 0° C between two molecular species having their λ_{max}s at 495 nm and at 466 nm (assigned to P-466 in Fig. 2); irradiation of the original pigment with orange light (> 560 nm) produced the P-466 and then irradiation of the P-466 with blue light (at 433 nm) reverted to the original pigment. This spectral shift is not due to photo-isomerization of the chromophore because almost no significant change in isomer composition of the chromophore was observed by a high-performance liquid chromatographic analysis (Fukada et al. 1983). Anyway, both components of this rhodopsin analogue exhibited small activation abilities which were comparable to that of bovine 11-*cis*-rhodopsin without irradiation (compare XX with XIX, presumably zero level). This indicates that the photo-isomerization of *cis* chromophore to all-*trans* form is the first essential event to the activation of the enzyme.

7. Accessibility of Hydroxylamine

Throughout these experiments, together with previous work (Ebrey et al. 1980), one should pay attention to the fact that the chromophores of natural visual pigments and artificial pigments which have some ability for the enzyme activation without irradiation were attacked by hydroxylamine in the dark. Chicken iodopsin, 7-*cis*-rhodopsin, 9-*cis*-retro-γ-rhodopsin (in the present work) and 11-*cis*-13-demethyl-rhodopsin (Ebrey et al. 1980) are the relevant cases. 11-*cis*-13-demethyl-rhodopsin specially impressed us because the 9-*cis* isomer which is resistant to hydroxylamine attack in the dark (Nelson et al. 1970) induced no dark activation of the enzyme (Ebrey et al. 1980). These common features could be simply interpreted as follows: Hydroxylamine can attack the chromophore only when the retinal binding site of opsin is widely expanded, i. e., the chromophores of artificial pigments which are attacked by hydroxylamine would sit in an "expanded" conformation of opsin, which may trigger the enzyme activation in the dark. In Fig. 2, the dimension of the opening of the retinal binding site of opsin is also drawn schematically.

8. Activation of Phosphodiesterase at Low Temperature

In a previous paper (Fukada and Yoshizawa 1981), we reported that the phosphodiesterase of dark-adapted frog rod outer segment membrane was

activated by irradiation at −4° C which is below the transition temperature of metarhodopsin II to metarhodopsin III or all-*trans*-retinal plus opsin (Matthews et al. 1963). Since the irradiation of rhodopsin at such a temperature, however, produces other isomers such as 7-*cis*- and 13-*cis*-retinals (Maeda et al. 1978, 1979), there is a possibility that such isomers other than all-*trans*-retinal (metarhodopsin II) might activate the enzyme. On the other hand, irradiation of rhodopsin at −196° C produces only 9-*cis*- (isorhodopsin) and all-*trans*-isomers (bathorhodopsin) (Maeda et al. 1978, 1979). Since both rhodopsin and isorhodopsin did not induce any phosphodiesterase activation, we can estimate which intermediate of bleaching of rhodopsin can activate the enzyme by irradiation of rhodopsin at −196° C and warming the sample to an appropriate temperature. In the present experiment, the dark-adapted frog rod outer segment membrane which had been irradiated at −196° C by light at 433 nm was warmed to −12° C and incubated at the same temperature. In order to prevent the sample from freezing, 33% glycerol was added to the preparation in the final concentration. As the concentration of glycerol increased, the phosphodies-

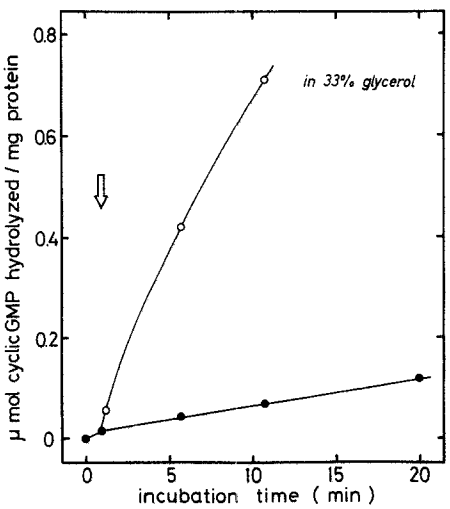


Fig. 1. Photo-activation of phosphodiesterase at −12° C. Dark-adapted frog rod outer segment membrane was mixed at 0° C with substrate solutions in 16 tubes in the dark. The final concentration of cGMP, GTP, or glycerol in each tube was 2 mM, 10 μM or 33% (v/v), respectively. Immediately after mixing, two of them were immersed in boiling water to stop the enzyme reaction (the first closed circle). One min after the mixing, the other aliquots were cooled to −196° C. Six aliquots of them were irradiated with blue flash (at 433 nm, duration: 100 ms), which converted 1% of rhodopsin to bathorhodopsin. The residual eight aliquots were kept in the dark. Irradiated and non-irradiated 14 aliquots were warmed to −12° C, where they were incubated for appropriate time. Then the enzyme reaction was stopped by immersing them into the boiling water. Setting the mixing time as 0, phosphodiesterase activities of dark-adapted (—●—) and irradiated (—○—) frog rod outer segment membranes were plotted against the period of the incubation at −12° C. The time when the aliquots were incubated at −196° C for about 20 min was omitted from the abscissa. The second closed circle represents the activity of the aliquot which was directly boiled from −196° C. This low activity permits us to neglect the incubation time at −196° C. The downward arrow indicates the time when the aliquots were irradiated. All points are averages of double measurements (± 5%)

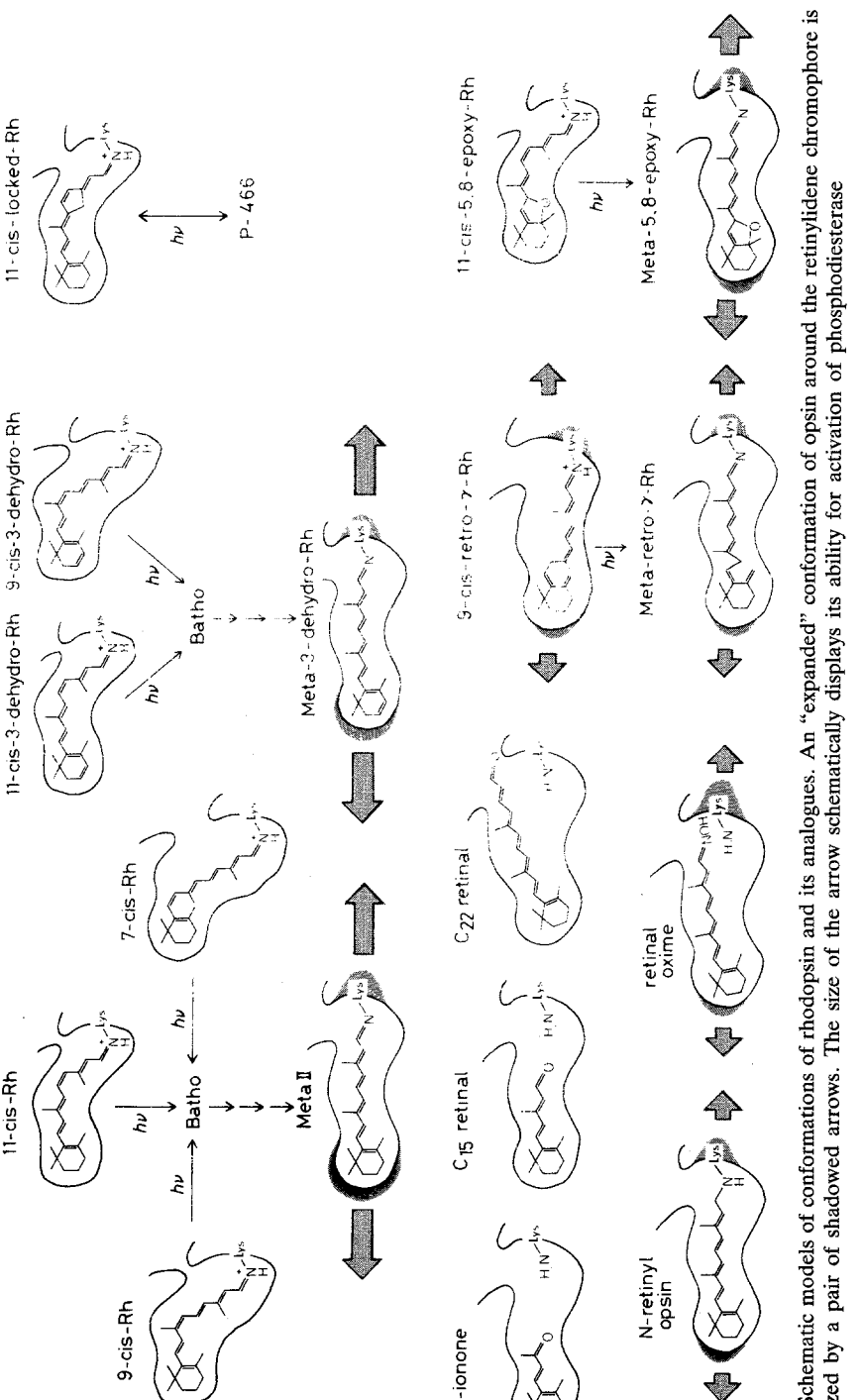


Fig. 2. Schematic models of conformations of rhodopsin and its analogues. An "expanded" conformation of opsin around the retinylidene chromophore is symbolized by a pair of shadowed arrows. The size of the arrow schematically displays its ability for activation of phosphodiesterase

terase activity of the membrane gradually decreased; nevertheless photo-activation of the enzyme was observed at 30° C in any concentration of glycerol below 66%. As shown in Fig. 1, the irradiation of the membrane increased the enzyme activity by about 9-fold. This fact indicates that the enzyme can be activated by metarhodopsin II or an earlier intermediate than metarhodopsin II.

This result would further support the speculation in Sect. 7, because metarhodopsin II is attacked by hydroxylamine (Matthews et al. 1963). Recently, metarhodopsin I has been reported to be attacked by hydroxylamine (Ratner et al. 1981), suggesting a possibility that metarhodopsin I would activate phosphodiesterase.

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