Activation of phosphodiesterase by chicken iodopsin

Yoshitaka Fukada and Tôru Yoshizawa*

Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa-Oiwakecho, Kyoto 606, Japan

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Activation of guanosine 3′,5′-cyclic monophosphate phosphodiesterase in outer-segment membrane of chicken retina was investigated. Irradiation of dark-adapted chicken outer segment membrane for bleaching of iodopsin increased the enzyme activity twice as much as that in the dark in the presence of GTP. Further irradiation of the sample for bleaching of rhodopsin in the membrane induced some additional activation of the enzyme. However, chicken iodopsin activated the enzyme in frog rod outer segment membrane without irradiation, while chicken rhodopsin did not. Irradiation of chicken iodopsin increased the enzyme activity twice as much as that in the dark.

Phosphodiesterase activation *Rhodopsin* *Iodopsin*

1. INTRODUCTION

Since guanosine 3′,5′-cyclic monophosphate (cGMP) phosphodiesterase in vertebrate rod cell can be activated by photo-bleaching of rhodopsin in the presence of both GTP and GTP-binding proteins, it has been proposed that cGMP may play a role of an intracellular transmitter in visual transduction process [1,2]. However, except for a brief description [3] that phosphodiesterase in ground squirrel cone displayed no photo-activation in the presence of ATP little information about the enzyme is available on the cone photoreceptor cell. On the other hand, a chicken cone visual pigment, iodopsin, has been characterized by its difference from rhodopsin in biochemical and photochemical behaviour [4–8]. To explore a possibility that the cone cell may have a phosphodiesterase to be activated by light in the presence of GTP, we have attempted to measure both the enzyme activity of chicken outer segment membrane and the activation ability of iodopsin and its photo-product on the enzyme in frog rod outer segment membrane.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

All manipulations, unless otherwise stated, were performed under infrared light (>800 nm) by use of a darkroom goggles (Type 5156, NV Optische Industrie, Delft).

2.1. *Preparation of chicken outer segment membrane*

Freshly decapitated chicken heads were dark-adapted for 6 h in a light-tight box. The retinas were isolated from the rear of the dissected eyeballs, and homogenized in 40% (w/w) sucrose-buffered with an isotonic solution (20 mM Tris–HCl, 120 mM NaCl, 50 mM KCl, 3 mM $MgCl_2$ and 2 mM dithiothreitol (pH 7.8): modified from [9]). The homogenate was filtered through a sheet of nylon cloth, followed by centrifugation at 100000 × *g* for 1 h. The paste which floated on the surface was collected and suspended in the isotonic solution. This suspension was used as a sample to measure phosphodiesterase activity in chicken outer segment membrane; it was provisionally named 'crude outer segment membrane'. Further purification of the membrane was done according to a slightly modified method [9]: The 'crude outer segment membrane' was laid on the top of a

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discontinuous sucrose gradient composed of 1.13 g/ml and 1.145 g/ml sucrose layers and then centrifuged at 40000 × *g* for 1 h in a rotor of a swinging bucket type. Purified outer segment membrane, floated at the interface between 1.13–1.145 g sucrose/ml layers, was collected and then irradiated at 0°C with orange light (> 540 nm) in the presence of neutralized hydroxylamine (50 mM) for complete bleaching of iodopsin and rhodopsin to yield photopsin (protein moiety of iodopsin) and scotopsin (protein moiety of rhodopsin). After being diluted with 2 vol. of the isotonic solution, the membrane suspension was spun down at 10000 × *g* for 20 min. The pellet thus obtained was washed ~10-times with the isotonic solution to remove the hydroxylamine and some water-soluble materials. After washing with distilled water, the pellet was lyophilized, suspended in light petroleum (b.p. 30–60°C) and then spun down at 10000 × *g* for 20 min. The washing with light petroleum was repeated 5 times. The final pellet was suspended in 18% (w/w) sucrose-buffered with the isotonic solution. This suspension was sonicated (Kaijo Denki Co., TA-4201 type sonicator) for 3 min (10 s, 18 times) and centrifuged at 5000 × *g* for 30 min to remove some black membraneous material. The final supernatant (purified opsin vesicle) was used for most experiments.

2.2. *Preparation of frog rod outer segment membrane*

Frog outer segment membrane was prepared from retinas of 12 h dark-adapted frogs (*Rana catesbeiana*) by 43% (w/w) sucrose flotation method [10] 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 has all the components necessary for the photo-activation of phosphodiesterase in the presence of GTP.

2.3. *Assays*

Phosphodiesterase activity was assayed using a reaction mixture composed of 4 mM 3′,5′-cyclic [^{3}H]GMP (1.5 Ci/mol), 20 μM GTP, 1 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM Tris–HCl (pH 8.0). The enzyme reaction was started by mixing the reaction mixture (10 μl) with membrane suspension (10 μl; containing 15–40 μg membrane protein) and incubated at 30°C for 1–4 min. The reaction was stopped by immersing the reaction tube in boiling water (90°C) for 1 min. The product of the enzyme reaction, 5′-GMP, was isolated from the reaction mixture by inorganic salt coprecipitation or by a thin-layer chromatography of polythyleneimine cellulose as in [10].

The molar extinction coefficients (ϵ) of chicken and frog rhodopsins were assumed to be the same as that of cattle rhodopsin ($\epsilon = 40600$ cm^{-1}.M^{-1} [11]) and ϵ of chicken iodopsin was described to be nearly equal to that of cattle rhodopsin [4].

The concentration of protein was determined as in [12] using bovine serum albumine as a standard.

3. RESULTS AND DISCUSSION

The 'purified opsin vesicle' (1.5 ml) containing photopsin and scotopsin was mixed with equimolar 11-*cis* retinal dissolved in ethanol (10 μl) and kept in the dark for 30 min at 10°C to regenerate iodopsin and rhodopsin. Then it was divided into two optical cuvettes. They were placed in the sample and reference compartments of a recording spectrophotometer (Shimadzu MSP-5000). The sample cuvette was irradiated with red light (at 670 nm) to bleach iodopsin only. After completion of the bleaching by successive irradiations (curves 1–6 in fig. 1), the sample was re-irradiated with orange light (> 550 nm) to bleach rhodopsin (curves 7 and 8). The λ_{max} positions of iodopsin and rhodopsin were located at 572 nm and 512 nm, respectively. They were close to those reported by microspectrophotometry [13] rather than those in digitonin solution [4]. In addition, the λ_{max} of iodopsin displayed a chloride ion effect; the absorption spectrum of Cl^--free form was located at shorter wavelength than that of Cl^--binding form [14]. In this report, the isotonic solution containing 176 mM Cl^- was used as a medium to obtain iodopsin in Cl^--binding form which is easily distinguishable from rhodopsin by a large difference in λ_{max}.

The phosphodiesterase activity of chicken outer segment membrane was measured in the presence of 10 μM GTP. Since the enzyme activity was greatly reduced in the course of the purification of the membrane, the 'crude outer segment membrane' (0.25 ml) was used. First, the phosphodiesterase activity of 10 μl aliquot of the sample was

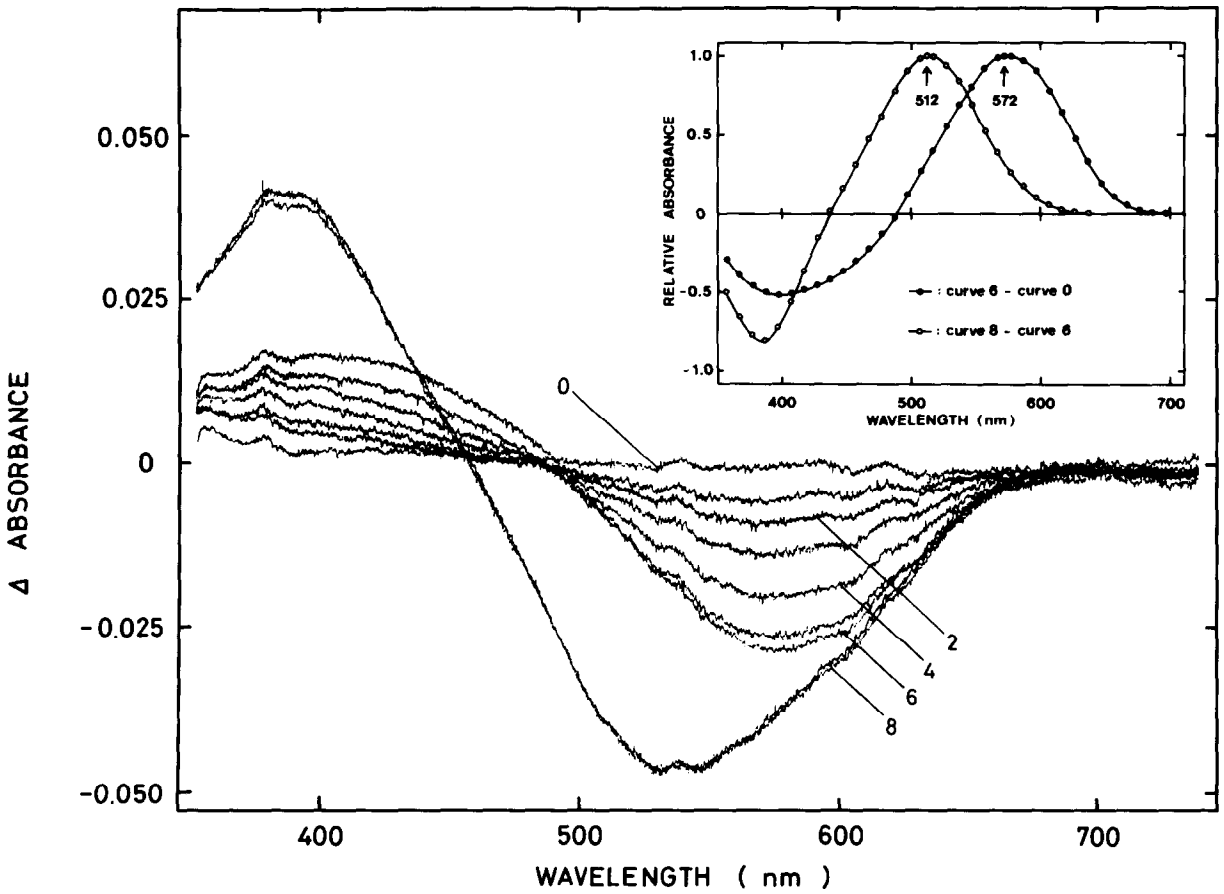

Fig. 1. Difference spectra of partial bleaching of iodopsin and rhodopsin in chicken outer segment. A mixture of iodopsin and rhodopsin regenerated from 11-*cis* retinal and the 'purified opsin vesicle' (curve 0) was irradiated with red light (at 670 nm) for successive periods of 0.5, 0.5, 1, 2, 4 and 8 min (curves 1–6) to bleach iodopsin, and then with orange light (>550 nm) for 0.5 and 0.5 min (curves 7,8) to bleach rhodopsin. In the inserted figure are shown the difference spectra between curves 0 and 6 and between curves 6 and 8, which correspond to iodopsin and rhodopsin, respectively. They were normalized at the absorption maxima. The irradiations and the spectrophotometric measurements were done at 0°C.

Table 1

Activation of phosphodiesterase in chicken outer segment membrane

	Irradiation	Phosphodiesterase activity (μmol cGMP hydrolyzed.min^{-1}.mg protein^{-1})[a]
I.	Non-irradiated	0.115
II.	Irradiated with red light (at 670 nm) at 0°C for 10 min	0.222
III.	Irradiated with red light and then orange light (>550 nm) at 0°C for 3 min	0.249

[a]The values are averages of double measurements (±5%)

measured in the dark (table 1, I). Then the remaining sample was irradiated at 0°C for 10 min with red light (at 670 nm) for bleaching of only iodopsin (see fig. 1). The enzyme activity of this sample (table 1, II) (another 10 μl aliquot) was then measured in the dark. It was roughly twice as large as that found for the untreated sample in the dark. Further irradiation of the sample at 0°C for 3 min with orange light (>550 nm) to bleach rhodopsin remaining in the sample (fig. 1) induced some additional activation of the enzyme (table 1, III). These results suggested that chicken cone outer segment has phosphodiesterase which can be activated by bleaching of iodopsin. However, another possibility is that bleached iodopsin might activate the rod phosphodiesterase in the sample because it is a mixture of rod and cone outer segments.

To examine this possibility, a mixing experiment was carried out: a purified chicken outer segment without phosphodiesterase activity was mixed with completely dark-adapted frog rod outer segment membrane containing rhodopsin, GTP-binding protein and phosphodiesterase. The 'purified opsin vesicle' was used as a starting material in order to eliminate the possibility that there might remain some active intermediate of rhodopsin and/or iodopsin in the preparation. The molar ratio of scotopsin to photopsin in the 'purified opsin vesicle' used in this experiment was ~1.4 on the average, according to 11-*cis* retinal titration. First, the 'purified opsin vesicle' was added to the dark-adapted frog rod outer segment membrane, and then the enzyme activity was measured in the dark. No enzyme activation was observed (—■—, fig. 2). Next, a limited amount of 11-*cis* retinal (80% in molar ratio of iodopsin content in the sample) was added to the 'purified opsin vesicle', resulting in regeneration of only iodopsin [9] because the rate of regeneration of iodopsin from photopsin and 11-*cis* retinal is ~500-times faster than that of rhodopsin from scotopsin and 11-*cis* retinal [4]. This sample contained 72% of the original iodopsin. In the dark, the sample induced some activation of the enzyme (—▲—). When it was irradiated with red light (at 670 nm) to bleach the iodopsin, the enzyme was activated by ~2-fold (—△—). Thus iodopsin displayed some additional activation of phosphodiesterase on photo-bleaching. The relatively small ratio of light-to-dark sample (that is, high activity of the dark sample) in the enzyme activity of chicken 'crude outer segment membrane' (I and II in table 1) could be ascribed to the activation ability of iodopsin in the dark. (In [15] using a ground squirrel retina, the concentration of cGMP in cone photoreceptor was 1–2 orders of magnitude greater than that in the other part of retina, but the cGMP level in the outer segment layer showed only a small decrease upon light adaptation. This observation may reflect the low activation ratio (light-to-dark) of phosphodiesterase in cone outer segment.)

Here, a sample containing both iodopsin and rhodopsin was prepared from the 'purified opsin vesicle' by addition of equimolar 11-*cis* retinal to the total content of iodopsin and rhodopsin (—●—). Almost the same ability for enzyme activation was observed as that for the partially regenerated iodopsin (—▲—), indicating that rhodopsin lacks the ability to activate the enzyme in the dark as was observed in the case of frog rhodopsin [16]. When the sample (—●—) was irradiated with red light (at 670 nm) at 0°C, it displayed a remarkable enzyme activation (—◑—), similar to that of the mixture of scotopsin and bleached iodopsin (—△—). The preparation containing the mixture of rhodopsin and iodopsin (—●—) was then irradiated with orange light (>550 nm) at 0°C in order to bleach both the visual pigments completely. A little additional activation of the enzyme was observed (—○—) by bleached rhodopsin. The low level of enzyme activation by bleaching of rhodopsin (compare —◐— with —○—) may be ascribed to a very small bleaching of rhodopsin by red light, by which the enzyme may have been activated to some extent. Thus, the true enzyme activation obtained by the bleaching of rhodopsin should be judged from the difference between (—○—) and (—△—).

Through the experiment mentioned above, we could not decide whether or not the chicken cone outer segment contained a photo-activated phosphodiesterase, because iodopsin has the capability of non-specific activation of the enzyme. In order to solve this problem, a new method for isolation of cone outer segment without any fusion with rod membrane must be developed.

4. CONCLUSIONS

(1) In the presence of GTP, the phos-

phodiesterase in chicken outer segment was almost completely activated by photo-bleaching of iodopsin. Further irradiation of rhodopsin brought about some additional activation of the enzyme. It seems probable that almost all the enzyme contained in chicken cone and/or rod outer segment(s) can be activated by bleached iodopsin; this conclusion is based on the finding that chicken iodopsin could activate the rod enzyme as observed in the mixing experiment of chicken iodopsin with the frog rod outer segment membrane.

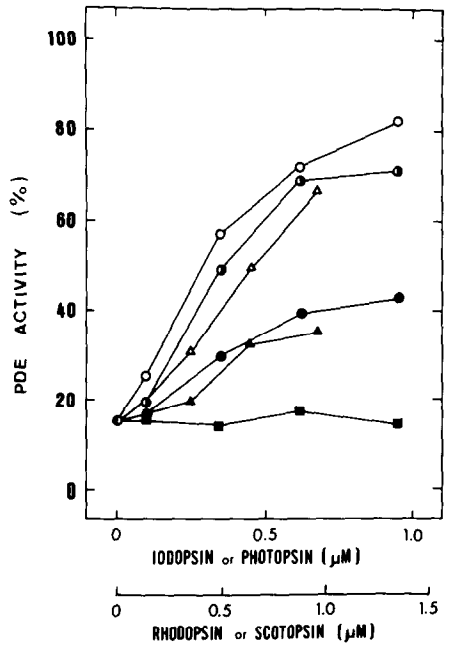

Fig. 2. Activation of frog phossphodiesterase by chicken iodopsin, rhodopsin and/or their photo-products. The 'purified opsin vesicle' containing photopsin and scotopsin (scotopsin/photosin = 1.43) was used as a starting material. 'Purified opsin vesicle' (—■—), partially regenerated iodopsin and scotopsin (—▲—), scotopsin and bleached iodopsin (—△—), regenerated iodopsin and rhodopsin (—●—), rhodopsin and bleached iodopsin (—◑—) or bleached iodopsin and bleached rhodopsin (—○—) was appropriately diluted and mixed with 9 vol. of dark-adapted frog rod outer segment membrane (1:9 mixing). Then the phosphodiesterase activity was measured in the dark at 30°C for 2 min. The activities were plotted against the concentrations of chicken iodopsin (or photopsin) and rhodopsin (or scotopsin) in the 1:9 mixture. The activities were expressed as percent of that of the irradiated (>550 nm, at 0°C for 1 min) frog rod outer segment membrane (cGMP hydrolyzed . min^{-1}. mg $protein^{-1}$ = 0.62 ± 0.08 μmol).

(2) Neither photopsin nor scotopsin in the chicken outer segment activate the frog rod phosphodiesterase.

(3) Chicken rhodopsin induces no activation of the frog rod phosphodiesterase, while iodopsin activates the enzyme to some extent without irradiation.

(4) Bleached iodopsin activates the frog rod phosphodiesterase. This activation is probably not due to bleaching of rhodopsin by red light, because the irradiation of the sample, which contained partially regenerated iodopsin without rhodopsin, also induced the enzyme activation to the same extent (—◑— and —△—, fig. 2).

(5) Bleached chicken rhodopsin can activate the frog rod phosphodiesterase to the same extent as that observed by bleached iodopsin.

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