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Stimulatory Effect of Cyanidin 3-Glycosides on the Regeneration of Rhodopsin

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Anthocyanins have been suggested to improve visual functions. This study examined the effect of four anthocyanins in black currant fruits on the regeneration of rhodopsin using frog rod outer segment (ROS) membranes. Cyanidin 3-glycosides, glucoside and rutinoside, stimulated the regeneration, but the corresponding delphinidins showed no significant effect. The formation of a regeneration intermediate was suggested to be accelerated by cyanidin 3-rutinoside. Their effects on the cGMP-phosphodiesterase activity in the ROS membranes were also investigated but found to be negligible. It was concluded that the major effect of anthocyanins in rod photoreceptors is on the regeneration of rhodopsin.

KEYWORDS: Rhodopsin; regeneration; 11-*cis*-retinal; anthocyanin; black currant

INTRODUCTION

Rhodopsin, which is a member of the G-protein-coupled receptor family, is localized on the lipid bilayers of disks laminated in the retinal rod outer segment (ROS). On light absorption, 11-*cis*-retinal, the chromophore of rhodopsin, is isomerized to *all*-*trans*-retinal (*1*). Rhodopsin consequently undergoes further conformational changes through a number of intermediates and triggers a series of reactions in the phototransduction cascade (*2*−*4*). One of the intermediates is metarhodopsin II, which activates >500 transducin molecules per second to amplify the light signal (*5*, *6*). Transducin then activates cGMP phosphodiesterase (PDE) to hydrolyze cGMP. Because cGMP opens a cGMP-gated cation channel, hydrolysis of cGMP by PDE induces the closure of the channel to hyperpolarize the cell. All of the phototransduction components recover to the original inactive state after light. Light-activated rhodopsin is inactivated by phosphorylation, subsequently dephosphorylated, and finally decomposed to *all*-*trans*-retinal plus opsin, the protein moiety of rhodopsin. Rhodopsin is then regenerated by binding of 11-*cis*-retinal to opsin.

Anthocyanins have been implicated to improve visual functions. Cyanidin, an aglycon of anthocyanin, has been suggested to have effects on the phototransduction cascade by activating (*7*) or inhibiting (*8*) PDE. Anthocyanins are also suggested to have effects on the regeneration of rhodopsin. A mixture of anthocyanins extracted from bilberry is known to improve night vision (*9*). The regeneration of rhodopsin has also been shown to be stimulated by bilberry extract (*10*). Although these studies suggest the effect of anthocyanins on the visual system, the detailed site of the action is not known. In addition, the molecular species responsible for the effects are not known. Because the crude extract contains chemicals other than anthocyanins and there are 15 species of anthocyanins, it has been difficult to identify the active species.

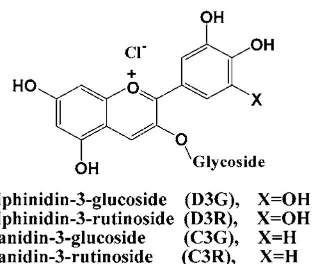

Figure 1. Structure of the four anthocyanins in black currant.

Black currant contains four anthocyanins (**Figure 1**): delphinidin 3-rutinoside (D3R), delphinidin 3-glucoside (D3G), cyanidin 3-rutinoside (C3R), and cyanidin 3-glucoside (C3G). In our previous studies, we successfully isolated and purified the four anthocyanins from black currant as crystals (*11*) and showed that they, as well as those from bilberry, have an improving effect on dark adaptation in humans (*12*).

In the present study, using the four forms of the black currant anthocyanins, first we examined the effect of anthocyanins on the PDE activation. Second, we examined their effects on the regeneration of rhodopsin.

MATERIALS AND METHODS

Reagents. Four crystalline components of black currant, D3R, D3G, C3R, and C3G, were purified according to the methods described

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previously (*11*). A standard buffer used in the following studies contained 115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.2 mM EGTA, and 10 mM HEPES, pH 7.5 (K-gluconate buffer). Other chemicals were of reagent grade.

Preparation of 11-*cis*-Retinal. Following the method of Shichida et al. (*13*), 11-*cis*-retinal was prepared with several modifications. Briefly, *all-trans*-retinal (Sigma) dissolved in acetonitrile (2 mg/mL) was isomerized by fluorescent irradiation at 4 °C for >24 h. The irradiated sample (500 μl) was loaded onto a normal-phase column (Supelcosil LC-Si, 250 × 21.2 mm, 5 μm, Supelco, Inc.) and eluted with diethyl ether/*n*-hexane (15:85) at 10 mL/min. Absorbance at 254 nm was monitored. Referring to the retention time of 9,13-di-*cis*-retinal (Sigma) as a standard (*14*), the eluate at 12.3 min was collected. Measurement of the UV−vis absorption spectrum of this fraction revealed two characteristic absorption maxima at 250 and 376 nm, which are specific to 11-*cis*-retinal. The purification was repeated to obtain a sufficient quantity of 11-*cis*-retinal. After the purification, the solvent was removed by evaporation, and the sample was dissolved in ethanol and stored at −80 °C until use. To avoid photoisomerization of the retinal sample, the manipulations were performed in the dark. The concentration of 11-*cis*-retinal was calculated on the basis of the molar extinction coefficient at 376 nm (ϵ = 25000).

Preparation of Frog ROS Membranes and Opsin Membranes. Preparation of frog ROS was carried out under infrared light using an infrared image converter (NVR 2015, NEC) (*15*). Frogs were dark-adapted for at least 3 h and then sacrificed by pith. After the eyeballs had been excised, the retinas were detached in the K-gluconate buffer and the ROS was brushed off the retinas into the buffer. The ROS was fragmented by passage through a syringe needle and suspended in the K-gluconate buffer (ROS membranes).

The opsin membranes used for the regeneration of rhodopsin were prepared from the ROS membranes. A 1 M NH_2OH solution (60 μL) was added to 1.2 mL of the ROS membranes, and rhodopsin was completely bleached on ice by white-light irradiation. To remove retinal-oxime, the bleached membranes were washed three times by ultracentrifugation (100000*g*) at 4 °C for 30 min. The final precipitate was then suspended in the K-gluconate buffer so that the opsin concentration was 5 μM (opsin membranes). The opsin membranes thus obtained were immediately frozen on liquid nitrogen and stored at −80 °C until use.

PDE Activity Measurement. Measurement of the PDE activity was carried out according to the method described previously (*15*). Briefly, a 200 μL portion of the ROS membranes was mixed with each of anthocyanin (final concentration = 10−50 μM), ATP (0.1 mM), GTP (0.1 mM), and cGMP (6 mM) at a low (1 nM) or high (10 μM) Ca^{2+} concentration. Rhodopsin concentration was 2−7 μM. When necessary, *S*-modulin (10 μM) was added to the membranes. Measurements were initiated by giving a weak light flash, and the pH decrease accompanied by hydrolysis of cGMP was monitored with a micro pH electrode (MI-410, Microelectrodes). After the pH decrease induced by a weak light flash had been recorded, a strong light was given to measure the maximum PDE activity. The data were digitized and stored in a hard disk and analyzed by Chart software (ADI Instruments).

Regeneration of Rhodopsin. Rhodopsin was regenerated by incubation of purified 11-*cis*-retinal with the opsin membranes. The reaction was initiated by the addition of 10 μL of 2 mM 11-*cis*-retinal to 1 mL of the opsin membranes at 4 °C. After incubation, the reaction was terminated by adding an equivolume of 40 mM cetyltrimethylammmonium bromide solution containing 20 mM NH_2OH. The amount of rhodopsin regenerated was determined by measuring the difference of the absorbance at 500 nm before and after bleach. All of the manipulations were carried out under infrared light. When necessary, a 10 μL solution containing 2 mM anthocyanin was added to 1 mL of the opsin membranes before the addition of 11-*cis*-retinal. Anthocyanins are known to degrade at high pH (*16*), but its effect was negligible in this study, which was carried out at 4 °C throughout. The absorption spectrum of an anthocyanin solution did not change significantly (<5%) for the period when the regeneration study was conducted (1 h).

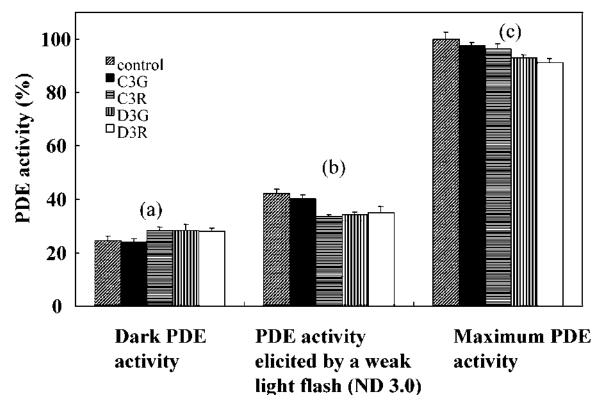


Figure 2. Effect of anthocyanins on PDE activation at low Ca^{2+} concentrations. PDE activities in the dark (a) and those elicited by a weak light flash (with an ND 3.0 filter) (b) and by a saturating light (c) were measured in the absence and presence of each of the four anthocyanins (10 μM each). Dark activities are included in the results shown in (b) and (c). The enzyme activity is expressed as a percentage of the maximum activity of the control, which did not include the anthocyanins. Values are the mean of four experiments, and vertical bars represent SEM.

RESULTS AND DISCUSSION

Effect of Anthocyanins on PDE Activity. In previous publications, the effects of anthocyanins on ROS PDE were controversial (*7*, *8*). Therefore, first we examined the effect of the four purified black currant anthocyanins on PDE. The PDE activities elicited by both a weak light flash and a saturating light were determined, and the relative activating efficiency by a weak light flash was calculated (**Figure 2**). The result showed that although the anthocyanins examined reduced slightly both the maximum PDE activity (**Figure 2c**) and the relative activating efficiency (**Figure 2b**), their effects were not so significant.

Because PDE activation is regulated by *S*-modulin in a Ca^{2+}-dependent manner (*17*), we then examined the effects of the four anthocyanins on the PDE activity in the presence of *S*-modulin at a high (10 μM) Ca^{2+} concentration. In agreement with previous studies (*18*), *S*-modulin increased the efficiency of PDE activation elicited by a weak light flash (compare the difference in the PDE activities between low and high Ca controls in **Figure 3a**) and prolonged the time required for 50% recovery of the PDE activation (**Figure 3b**). The four anthocyanins slightly reduced the efficiency of the PDE activation caused by a weak light flash (**Figure 3a**), but the maximum PDE activity in addition to the dark PDE activity (not shown) and the time required for 50% recovery (**Figure 3b**) were indistinguishable from those of the high Ca^{2+} controls. From these results, we concluded that there might be a small effect of the four anthocyanins on the PDE activation, but the effect is very small even at concentrations of 10−50 μM. Because anthocyanin concentrations are much lower in the plasma (*19*), the in vivo effect of anthocyanins on PDE is negligible.

The above finding contrasts with the previous studies that 10 μM concentrations of anthocyanidins (aglycon of anthocyanin) activate (*7*) or inhibit (*8*) this enzyme. One of the reasons for this inconsistency may possibly be due to the difference in the presence (this study) and absence (previous studies) of saccharide modifications on anthocyanins. It is noteworthy that anthocyanins in plasma and in any organs are usually glycosylated. Another reason might be due to the difference in the light condition used in the measurements. We measured the PDE activity in real time after activation by a light flash. Previous

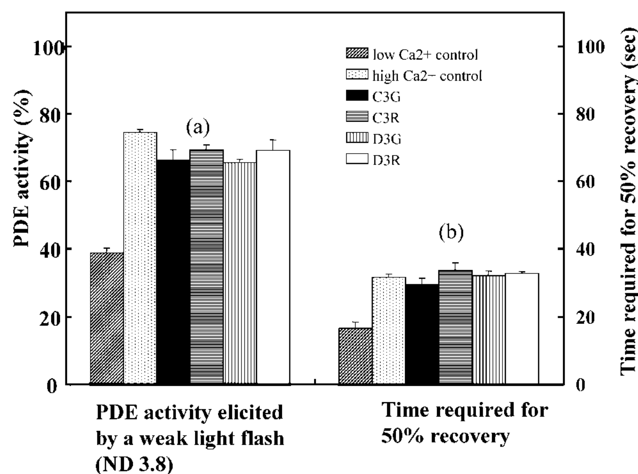


Figure 3. Effect of anthocyanins on the sensitivity (a) and lifetime (b) of PDE at high Ca^{2+} concentrations. PDE activities were measured in the presence of *S*-modulin (10 μM) at a high Ca^{2+} concentration (10 μM). Control measurements were done in the absence of the anthocyanins but in the presence of *S*-modulin at high (10 μM) and low (1 nM) Ca^{2+} concentrations. Dark activities were subtracted to show the net effect of the light. Values are the mean of two experiments, and vertical bars represent the range of the variation.

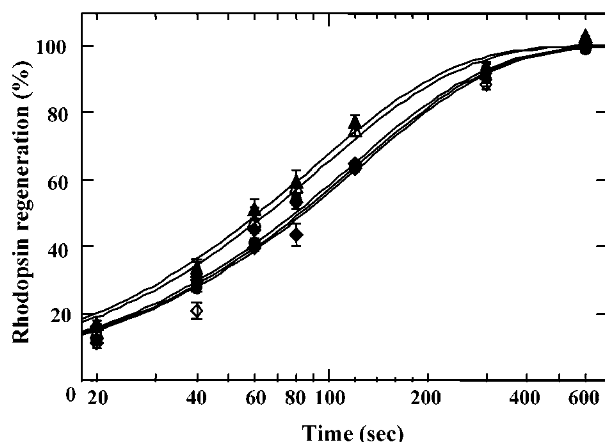


Figure 4. Effect of anthocyanins on the time course of rhodopsin regeneration, which was measured in the absence and presence of each of the four anthocyanins: (●) control; (◇) D3G; (◆) D3R; (△) C3G; (▲) C3R. The data points were fitted to an exponential function of 1 − exp(-t/τ): control, $\tau = 114$ s ($R^2 = 0.997$); D3G, $\tau = 122$ s ($R^2 = 0.988$); D3R, $\tau = 119$ s ($R^2 = 0.993$); C3G, $\tau = 94$ s ($R^2 = 0.994$); C3R, $\tau = 89$ s ($R^2 = 0.994$). Values are the mean of nine experiments, and vertical bars represent SEM.

studies were done at several minutes after full bleaching of rhodopsin, so this difference in the preparation may explain the difference in the results.

Effect of Anthocyanins on Rhodopsin Regeneration. Regeneration of rhodopsin was measured for 10 min after the addition of 11-*cis*-retinal with or without purified anthocyanins. **Figure 4** shows the time course of the regeneration without (control, ●) or with anthocyanins (D3G, ◇; D3R, ◆; C3G, △; C3R, ▲). The rhodopsin regeneration was fitted to an exponential function (solid lines). **Figure 4** shows that D3G and D3R (diamonds) showed no significant effects on rhodopsin regeneration but that C3G and C3R (triangles) stimulated the regeneration of rhodopsin. The result clearly demonstrated that the cyanidin form of anthocyanin has the activity to accelerate the regeneration of rhodopsin.

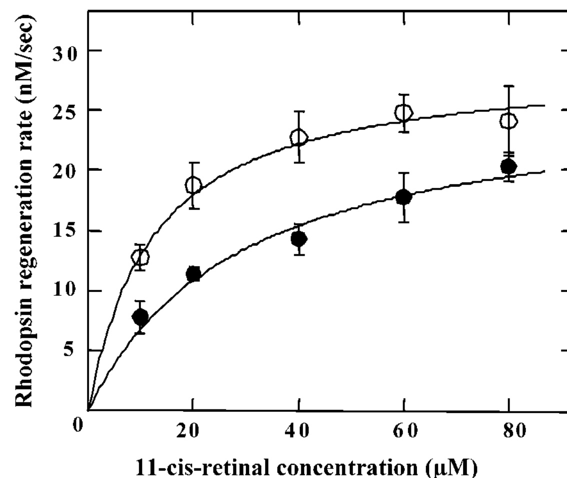


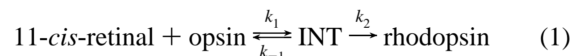
Figure 5. Effect of C3R on the initial velocity of rhodopsin regeneration at various concentrations of 11-*cis*-retinal. Initial velocity of rhodopsin regeneration was determined for 40 s in the presence (○) and absence (●) of C3R at various concentrations of 11-*cis*-retinal. The data points were fitted with eq 2 to determine the rate constants in the regeneration reaction (see text). Values are the mean of six experiments, and vertical bars represent SEM.

Table 1. Kinetic Parameters in the Regeneration of Rhodopsin

	control[a]	+ C3R[a]
k_2 (s^{-1})	1.0×10^{-2}	1.1×10^{-2}
K_m (M)	2.6×10^{-5}	1.1×10^{-5}

[a] $R^2 = 0.989$ (control); 0.995 (+ C3R).

The reaction scheme of the regeneration can be assumed as in (*20*)



$$11\text{-}cis\text{-retinal} + \text{opsin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{INT} \xrightarrow{k_2} \text{rhodopsin} \quad (1)$$

where k_1, k_{-1}, and k_2 are the rate constants and INT is a regeneration intermediate.

The solution of the above reaction under the condition of excess 11-*cis*-retinal is

$$v_{obsd} = k_2[\text{opsin}][11\text{-}cis\text{-retinal}]/(K_m + [11\text{-}cis\text{-retinal}]) \quad (2)$$

where v_{obsd} is the observed velocity of the regeneration of rhodopsin at a certain concentration of 11-*cis*-retinal and $K_m = (k_{-1} + k_2)/k_1$.

Equation 2 reveals that the observed velocity of the regeneration is dependent on the concentration of 11-*cis*-retinal, and its dependency is determined by k_1, k_{-1}, and k_2. To estimate the values of these rate constants, we measured the initial velocities of the regeneration of rhodopsin to determine v_{obsd} as a function of the concentration of 11-*cis*-retinal. The measurement was performed also in the presence of C3R, the major cyanidin glycoside in black currant (**Figure 5**). We fitted the result with eq 2 to obtain the values of k_2 and K_m in the presence and absence of C3R (solid lines in **Figure 5**).

The result (**Table 1**) showed that C3R did not affect k_2, but instead decreased the K_m value by 2.4-fold from that of the control. The decrease in the K_m value can be accounted for by an increase in k_1 or a decrease in k_{-1}. Because of the scatter of the data, it was difficult to determine which was the case. However, it is evident that the production of a regeneration intermediate (INT) is facilitated in the presence of C3R.

Effect of Anthocyanins on Dark Adaptation. Anthocyanins first gained interest because they possess an improving effect on visual acuity (*21*). Since then, several studies of anthocyanins on vision, such as dark adaptation (*9*), rhodopsin regeneration (*10*), transient refractive alternation (*12*), have been reported. The improvement of dark adaptation (*9*) can be accounted for by at least two reasons. Because dark adaptation accompanies the increase in the light sensitivity of a rod, one possibility is that there might be an effect of anthocyanins on the phototransduction to increase the efficiency of cGMP hydrolysis. However, our present study showed that they have only a limited effect on PDE activation even in the presence of *S*-modulin (**Figures 2** and **3**). Our result therefore indicated that the stimulatory effect of the four anthocyanins on hydrolysis of cGMP does not seem to be plausible.

The other possibility is the enhancement of the regeneration of rhodopsin. This possibility was suggested in a previous study (*10*), but anthocyanins used in that study were partial components of plant preparations and the contents were below 25% (w/w). For this reason, the effective components of anthocyanins were not certain. In the present study, we used purified anthocyanins to show distinct effects of some of the anthocyanins on the regeneration of rhodopsin (**Figure 4**) and found that the cyanidin form accelerates the rhodopsin regeneration most probably through enhancement of the formation of an intermediate, INT (**Figure 5** and **Table 1**).

Recent studies have indicated that even after bleach, opsin maintains its ability to activate the phototransduction mechanism to a small extent and reduces the intracellular Ca^{2+} concentration, which results in the decrease in the light sensitivity of a rod (*4*). Therefore, the acceleration of the regeneration of rhodopsin by anthocyanins is expected to accelerate the recovery of the light sensitivity of a rod to a high dark level. This mechanism probably explains the improvement of night vision by anthocyanins extracted from bilberry (*9*).

The anthocyanin concentration used in the regeneration study (20 μM) is considerably higher than that in the plasma (1 μM) (*19*), and thus the effect of the cyanidin form under in situ conditions might be smaller than that observed in the present study. However, the effect might be very important under very dark conditions, because the acceleration of regeneration of a single bleached rhodopsin molecule among a limited number of bleached visual pigments will affect the light sensitivity of a rod significantly. This possibility, however, needs further studies to be proven.

In the present study, the cyanidin form of anthocyanin was found to be active but the delphinidin form was not. There is a structural difference between cyanidin 3-glycosides and corresponding delphinidin glycosides (**Figure 1**). Because 11-*cis*-retinal is a nonpolar substance, the cyanidin form might be more effective than the delphinidin form that is less hydrophobic than cyanidin glycosides.

ABBREVIATIONS USED

ROS, rod outer segment; PDE, phosphodiesterase; D3R, delphinidin 3-rutinoside; D3G, delphinidin 3-glucoside; C3R, cyanidin 3-rutinoside; C3G, cyanidin 3-glucoside; INT, intermediate.

LITERATURE CITED

(1) Yoshizawa, T.; Wald, G. Pre-lumirhodopsin and the bleaching of visual pigments. *Nature* **1963**, *197*, 1279−1286.

(2) Burns, M. E.; Baylor, D. A. Activation, deactivation, and adaptation in vertebrate photoreceptor cells. *Annu. Rev. Neurosci.* **2001**, *24*, 779−805.

(3) Menon, S. T.; Han, M.; Sakmar, T. P. Rhodopsin: structural basis of molecular physiology. *Physiol. Rev.* **2001**, *81*, 1659−1688.

(4) Fain, G. L.; Matthews, H. R.; Cornwall, M. C.; Koutalos, Y. Adaptation in vertebrate photoreceptors. *Physiol. Rev.* **2001**, *81*, 117−151.

(5) Emeis, D.; Kuhn, H.; Reichert, J.; Hofmann, K. P. Complex formation between metarhodopsin II and GTP-binding protein in bovine photoreceptor membranes leads to a shift of the photoproduct equilibrium. *FEBS Lett.* **1982**, *143*, 29−34.

(6) Tachibanaki, S.; Imai, H.; Mizukami, T.; Okada, T.; Imamoto, Y.; Matsuda, T.; Fukada, Y.; Terakita, A.; Shichida, Y. Presence of two rhodopsin intermediates responsible for transducin activation. *Biochemistry* **1997**, *36*, 14173−14179.

(7) Virmaux, N.; Bizec, J. C.; Nullans, G.; Ehret, S.; Mandel, P. Modulation of rod cyclic GMP-phosphodiesterase activity by anthocyanin derivatives. *Biochem. Soc. Trans.* **1990**, *18*, 686−687.

(8) Ferretti, C.; Magistretti, M. J.; Robotti, A.; Ghi, P.; Genazzani, E. *Vaccinium myrtillus* antocyanosides are inhibitors of cAMP and cGMP phosphodiesterase. *Phamacol. Res. Commun.* **1988**, *20*, 150.

(9) Scialdone, D. L'Azione delle antocianine sul senso luminoso. *Ann. Oftalmol. Clin. Ocul.* **1966**, *92*, 43−51.

(10) Bastide, P.; Rouher, F.; Tronche, P. Rhodopsin et anthocyanosides. *Bull. Soc. D'Ophtalmologie Fr.* **1968**, *9*, 801−807.

(11) Matsumoto, H.; Hanamura, S.; Kawakami, T.; Sato, Y.; Hirayama, M. A preparative-scale isolation of four anthocyanin components in blackcurrant (*Ribes nigrum* L.) fruits. *J. Agric. Food Chem.* **2001**, *49*, 1541−1545.

(12) Nakaishi, H.; Matsumoto, H.; Tominaga, S.; Hirayama, M. Effects of blackcurrant anthocyanosides intake on dark adaptation and VDT work-induced transient refractive alternation in healthy humans. *Altern. Med. Rev.* **2000**, *5*, 553−562.

(13) Shichida, Y.; Imai, H.; Iwamoto, Y.; Fukada, Y.; Yoshizawa, T. Is chicken green-sensitive cone visual pigment a rhodopsin-like pigment? A comparative study of the molecular properties between chicken green and rhodopsin. *Biochemistry* **1994**, *33*, 9040−9044.

(14) Knowles, A.; Priestley, A. The preparation of 11-*cis*-retinal. *Vision Res.* **1978**, *18*, 115−116.

(15) Tachibanaki, S.; Tsushima, S.; Kawamura, S. Low amplification and fast visual pigment phosphorylation as mechanisms characterizing cone photoresponses. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14044−14049.

(16) Cmeminat, A.; Brouillaed, R. PMR Investigation of 3-*O*-(β-D-glucosyl)-malvidin structural transformation in aqueous solutions. *Tetrahedron Lett.* **1986**, *27*, 4457−4460.

(17) Kawamura, S.; Murakami, M. Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. *Nature* **1991**, *349*, 420−422.

(18) Kawamura, S. Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by *S*-modulin. *Nature* **1993**, *362*, 855−857.

(19) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *J. Agric. Food Chem.* **2001**, *49*, 1546−1551.

(20) Hanselman, A. R.; Cusanovish, M. A. Characterization of the recombination reaction of rhodopsin. *Biochemistry* **1976**, *15*, 5321−5325.

(21) Jayle, G. E.; Aubert, L. Action des glycosides d'anthocyanes sur la vision scopique et mesopique du sujet normal. *Therapie* **1964**, *19*, 171−185.

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