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EFFECT OF DIGITONIN CONCENTRATION ON REGENERATION OF CATTLE RHODOPSIN

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Summary

The effect of digitonin concentration on the regeneration of cattle rhodopsin was studied. The second-order rate constant (k_2) of the regeneration of rhodopsin was measured under pseudo first-order conditions of 11-*cis*-retinal excess in the presence of digitonin at various concentrations, showing that above the critical micelle concentration of digitonin the regeneration is inhibited more than that below the critical micelle concentration. Far below the critical micelle concentration (<0.01%), k_2 reaches a constant value, 7500 $M^{-1} \cdot s^{-1}$, at 25°C and pH 7.0. Far above the critical micelle concentration (2%), k_2 is 50 $M^{-1} \cdot s^{-1}$, which agrees with that reported previously by Wald and Brown (Wald, G. and Brown, P.K. (1956) Nature 177, 174—176). The regeneration of rhodopsin in rod outer segments was also measured, showing that k_2 in rod outer segments is similar (5600 $M^{-1} \cdot s^{-1}$) to that in digitonin below the critical micelle concentration.

The inhibition of the regeneration of rhodopsin by β-ionone ($8.71 \cdot 10^{-5}$ M) diminishes above the critical micelle concentration of digitonin. The activation energies of the regeneration of rhodopsin above and below the critical micelle concentration of digitonin are approx. 22 kcal/mol. These results suggest that the dissociation constant of an 11-*cis*-retinal · opsin complex (K_m), in which 11-*cis*-retinal and opsin are postulated to bind each other through a β-ionone ring-binding site in opsin, increases above the critical micelle concentration of digitonin more than that below the critical micelle concentration, and that under both conditions the rate-determining step is the slow formation process of the retinylidene-protonated Schiff base in rhodopsin.

Other activation parameters were estimated on the regeneration of rhodopsin above and below the critical micelle concentration of digitonin, which were discussed in the light of the above-mentioned mechanism. The dark adaptation

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process in vivo, which reflects the regeneration of rhodopsin, was discussed, and an existence of a pool of a precursor of 11-*cis*-retinal was suggested.

Introduction

Kinetic studies of regeneration of rhodopsin from 11-*cis*-retinal and opsin have been done in aqueous digitonin [1,2], because rhodopsin does not regenerate in any other detergents except cholic acid [3] or recently reported alkylglucosides [4]. In a previous paper [5], it was reported that the lower the concentration of digitonin, the faster the regeneration of cattle rhodopsin. In the present paper, the effect of the digitonin concentration on the kinetics of regeneration of cattle rhodopsin are studied more extensively. The rate constant of regeneration of cattle rhodopsin measured in digitonin micelles is compared with that in fragments of rod outer segment. The mechanism of the inhibition of the rhodopsin regeneration by digitonin and the dark adaptation process in vivo are also discussed.

Materials and Methods

Preparation of rod outer segments and opsin. Rod outer segments were prepared from dark-adapted cattle retinas by the usual method [6]. They were then suspended in 10 mM NH_2OH dissolved in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) and irradiated with a 500 W Na lamp to bleach the rhodopsin completely. After washing more than 10 times with 10 mM HEPES buffer to remove NH_2OH and then several times with distilled water, the rod outer segments were suspended in buffer and used for the kinetic studies. For preparation of opsin a part of the rod outer segments was lyophilized. Then the lyophilized rod outer segments were washed several times with chilled light petroleum (b.p. 40—60° C). Then opsin was extracted with 0.77% digitonin in 10 mM HEPES buffer (pH 7.0). The purity of opsin which can be defined as A_{280nm}/A_{500nm} of the regenerated rhodopsin ranged from 2.1 to 2.5.

Digitonin (1 g) purchased from Sigma (Lot. No. 35C-0169) was dissolved in distilled water (50 ml) by boiling and then left for 1 month at room temperature. Meanwhile some digitonin precipitated. The supernatant was used for extracting opsin. The digitonin concentration in the supernatant was determined by measuring the dry weight of the digitonin solution. Thus we estimated the digitonin solution at 0.77%. In the course of the preparation of this paper, Bridges [7] reported essentially the same method for preparation of digitonin solution.

All the buffer used was deoxygenated by bubbling nitrogen gas to avoid the oxidation of lipids [8]. The rod outer segments and the extracted opsin were stored under nitrogen atmosphere at 0°C or —20°C, respectively. The rod outer segments was used within 1 week after the peparation, opsin within several weeks. The rate constants of regeneration of rhodopsin and the amount of active opsin in the sample did not change significantly under these conditions.

Assay of regeneration of rhodopsin. Two assay methods for the measurement of the regeneration of rhodopsin were used. In Method I the regeneration

was performed in an optical cell placed in a recording spectrophotometer (Hitachi 323) at 25°C. Increase of absorbance at 530 nm was recorded after addition of 11-*cis*-retinal. The reaction mixture (total volume, 2.0 ml) contained 1.02 μM of opsin and 7.01 μM of 11-*cis*-retinal in final concentrations in 10 mM HEPES buffer (pH 7.0). In Method II the reaction mixture (total volume, 5.0 ml) contained 1.26 μM of opsin and 7.01 μM of 11-*cis*-retinal in final concentrations in 10 mM HEPES buffer (pH 7.0). The aliquot (250 μl) of the reaction mixture was mixed with the same volume of 600 mM NH_2OH dissolved in 2% Triton X-100 at each time using a rapid mixing device (Union Giken Model MX-7-01) temperature controlled at 25°C. After stopping the reaction, the total amount of rhodopsin was determined by measuring the difference spectrum between before and after bleaching. In both methods the reactions were started by adding 11-*cis*-retinal dissolved in ethanol. In all the cases the final concentrations of ethanol were below 1%. We confirmed that ethanol did not affect the regeneration, at least up to 3%. The 11-*cis*-retinal was 5—7 times excess to opsin. Thus the time courses of regeneration fitted to a pseudo first-order kinetics in all measurements.

The regeneration of rhodopsin in rod outer segments were measured by Method II using the rod outer segments preparation instead of the extracted opsin.A small amount of rod outer segments, equivalent to approx. 1 μM of opsin in final concentration, was dispersed in HEPES buffer at the relatively low concentration of 10 mM.

The effect of temperature on the regeneration of rhodopsin was measured only by Method I. The HEPES buffers (10 mM) were adjusted to pH 7.0 at each temperature.

The concentrations of digitonin were adjusted by dilution of 0.77% digitonin in 10 mM HEPES buffer with the same buffer. The inhibition of the regeneration reaction of rhodopsin by β-ionone was measured as described previously [5]. Percentage inhibition was defined as follows;

$$\text{percentage inhibition} = \frac{(k_{\text{control}} - k_{\text{inhibited}})}{k_{\text{control}}} \times 100,$$

where k_{control} is the pseudo first-order rate constant without β-ionone and $k_{\text{inhibited}}$ is that with β-ionone.

The 11-*cis*-retinal solution in ethanol was freshly prepared from 11-*cis*-retinal crystals. A high performance liquid chromatographic analysis of this sample gave a single peak. Titration of this sample with opsin (opsin test [6]) showed the purity higher than 98%. The container of the stock solution of 11-*cis*-retinal (ethanol solution) was rinsed by 1 M NaOH to avoid the hemiacetal formation [9]. During the experiments no hemiacetal formation occurred, which was checked by ultraviolet absorption spectrum. The concentrations of 11-*cis*-retinal (ϵ_m: 24 900 in ethanol) and cattle rhodopsin (ϵ_m: 42 000 [10]) were determined spectrophotometrically.

Results

Effect of digitonin concentration on the regeneration kinetics of the rhodopsin

Fig. 1a shows time courses of regeneration of cattle rhodopsin at different

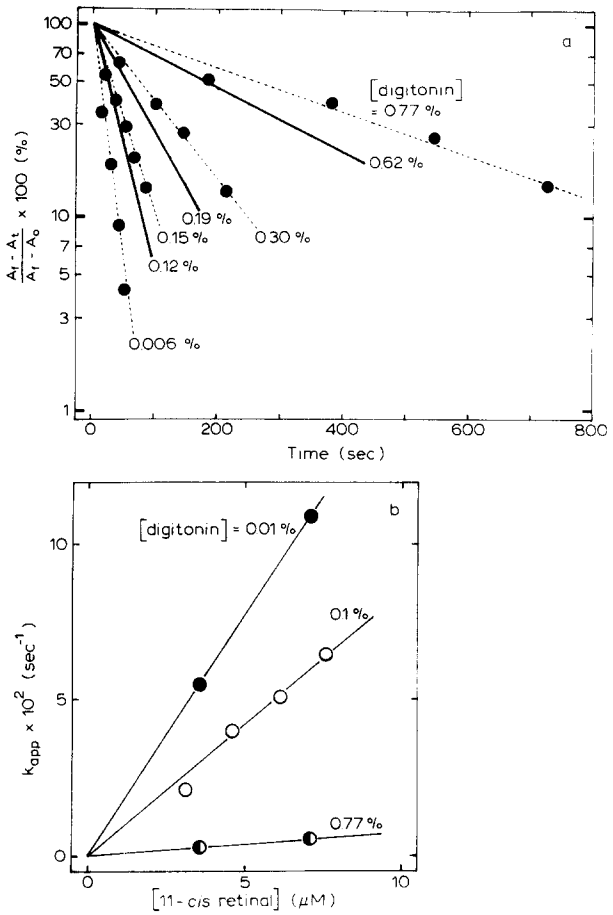

Fig. 1. Regeneration of cattle rhodopsin at different concentrations of digitonin. (a) Pseudo first-order kinetics of the regeneration of rhodopsin. Solid lines; measured by Method I. The reaction mixtures contained 1.02 μM of opsin and 7.01 μM of 11-*cis*-retinal in final concentrations in 2 ml HEPES buffer. The concentrations of digitonin are indicated in the figure. Dotted lines; measured by Method II. The reaction mixtures contained 1.26 μM of opsin and 7.01 μM of 11-*cis*-retinal in final concentrations in 5 ml HEPES buffer. All the reactions were done at 25°C and pH 7.0. A_f is the final absorbance at 530 nm (Method I) or 500 nm (Method II) when the regeneration was completed and A_t is the absorbance at 530 nm (Method I) or 500 nm (Method II) at time, t. A_0 is the initial absorbance at 530 nm (Method I) or 500 nm (Method II). (b) Linearity of the apparent pseudo first-order rate constant (k_{app}) against concentration of 11-*cis*-retinal under various concentrations of digitonin. The concentrations of digitonin are indicated in the figure. In a series of the measurements Method I was used and the concentration of 11-*cis*-retinal was changed under a constant concentration (1.02 μM) of opsin.

concentrations of digitonin, which performed under pseudo first-order conditions of 11-*cis*-retinal excess. Solid lines in Fig. 1a show the results measured by Method I in which the increment of absorbance at 530 nm was recorded. When NH_2OH was added to the reaction mixture after the completion of the regeneration of rhodopsin, no decrease of the absorbance at 530 nm was observed. Therefore we can measure the regeneration of rhodopsin without any contamination by formation of a retinylidene Schiff base. Dotted lines in Fig. 1a show the results measured by Method II in which the amount of the regenerated rhodopsin were determined at each time from the difference of

absorbance at 500 nm before and after complete bleaching in the presence of NH_2OH. The slopes of these straight lines gave apparent pseudo first-order rate constant (k_{app}) of the regeneration of rhodopsin at different concentrations of digitonin. Fig. 1b shows the linearity of k_{app} under these different concentrations of digitonin. One can calculate second-order rate constants (k_2) under these conditions by the following equation;

$$k_2 = k_{app}/[11\text{-}cis\text{-retinal}]. \quad (1)$$

Then k_2 of the regeneration against the digitonin concentrations were plotted in Fig. 2a. Both of the k_2 values from Method I (●) and Method II (○) coincide with each other as represented by an s-shaped line in the figure. The arrow shows the critical micelle concentration of the digitonin, which we determined in another experiment by measuring the sudden increase of scattering at 700 nm light aginst the concentration (data not shown). The critical micelle concentration of digitonin (0.09% at 25°C and pH 7.0) is almost identical with the value reported by Bridges [11], although digitonin may differ in impurity among different companies or batches.

Therefore we conclude that the regeneration of cattle rhodopsin is inhibited under the condition that the digitonin concentration is higher enough to form

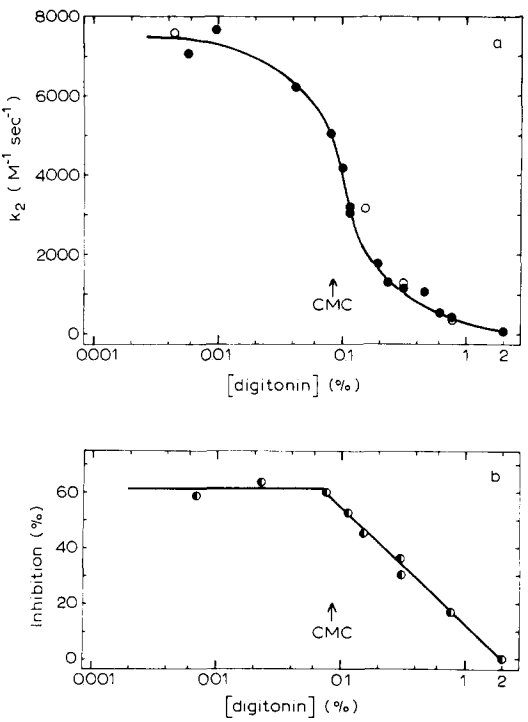

Fig. 2. Effect of the concentration of digitonin on the second-order rate constant (k_2) and on the inhibition by β-ionone of the regeneration of rhodopsin. (a) Dependence of k_2 on the digitonin concentration. Filled circles (●): data obtained from Method I. Open circles (○): data obtained from Method II. (b) Effect of the concentration of digitonin upon the competitive inhibition of the regeneration of rhodopsin by β-ionone ($8.71 \cdot 10^{-5}$ M). CMC indicates the critical micelle concentration of digitonin.

micelles. At 2% digitonin k_2 (50 $M^{-1} \cdot s^{-1}$) well coincides with that (43 $M^{-1} \cdot s^{-1}$) reported by Wald and Brown [2] within experimental error. Far below the critical micelle concentration (<0.008%) k_2 reaches a constant value, 7500 $M^{-1} \cdot s^{-1}$.

Effect of digitonin concentration on inhibition of the regeneration of rhodopsin by β-ionone

The effect of digitonin concentration on the competitive inhibition of the regeneration of rhodopsin by β-ionone [5] was examined (Fig. 2b). As the concentration of digitonin increases above the critical micelle concentration, the inhibition decreases and reaches zero at 2% digitonin. Since β-ionone was preincubated with opsin for the period that the control reaction without β-ionone was completed, equilibrium of β-ionone with opsin was believed to have been established in the experiment.

Regeneration of rhodopsin in rod outer segments

The regeneration of rhodopsin in rod outer segments was measured using Method II. The merit of Method II is that one can directly measure the regenerated rhodopsin without any contamination from random retinylidene Schiff bases. The difference of absorbance at 500 nm (ΔA_{500nm}) between before and after bleaching represents the amount of rhodopsin regenerated. Measurements were done at different concentrations of 11-*cis*-retinal. The results are summarized in Fig. 3a. The reaction when the concentration of 11-*cis*-retinal equals to 1.44 μM did not fit a pseudo first-order kinetics, because 11-*cis*-retinal was not in excess over opsin. Plotting the initial part of the reaction, one can get a straight line and estimate k_{app}. Other reactions were done under pseudo first-order conditions where 11-*cis*-retinal was in excess. Thus the kinetics fitted straight lines to regeneration levels of more than 90% (except for 4.32 μM retinal where regeneration over 70% was not measured).

As shown in Fig. 3b, k_{app} displayed linearity against 11-*cis*-retinal at least up to approx. 10 μM. The second-order rate constant (k_2) for the regeneration of rhodopsin in rod outer segments in this case was found to be 5600 $M^{-1} \cdot s^{-1}$.

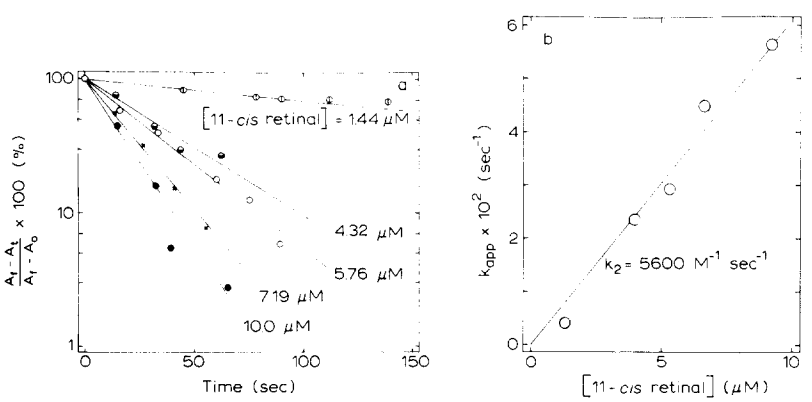

Fig. 3. Regeneration of cattle rhodopsin in rod outer segments. (a) Pseudo first-order kinetics at various concentrations of 11-*cis*-retinal. A_f, A_t and A_0 are the same as in Fig. 1a. (b) Linearity of k_2 against concentration of 11-*cis*-retinal.

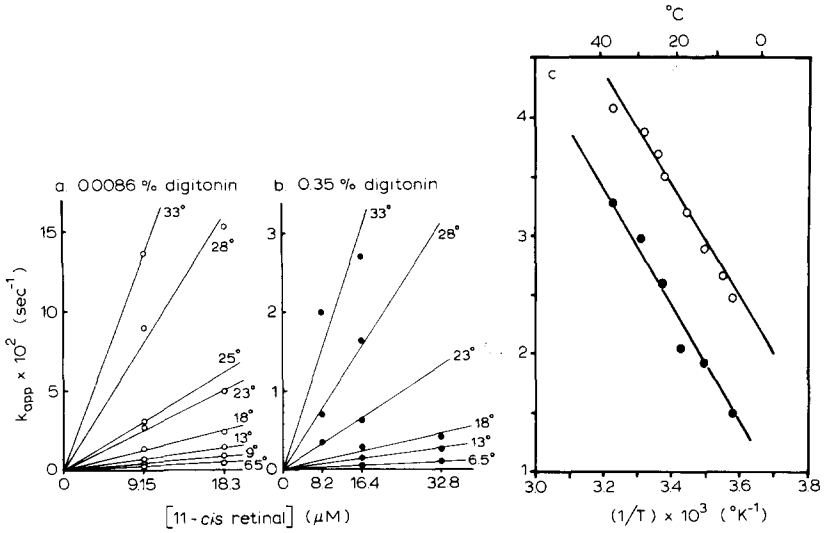

Fig. 4. Effect of temperature on k_2 below (a) and above (b) the critical micelle concentration of digitonin. (a) 0.0086% digitonin (b) 0.35% digitonin. The temperatures are indicated in the figure. (c) Arrhenius plot of k_2. ○——○, 0.0086% digitonin; ●——●, 0.35% digitonin.

Effect of temperature on the regeneration of rhodopsin below and above the critical micelle concentration of digitonin

To obtain activation parameters of the regeneration of rhodopsin the effect of temperature on the regeneration below and above the critical micelle concentration of digitonin were examined. Figs. 4a and 4b show the dependence of k_{app} on 11-*cis*-retinal at various temperatures measured in 0.0086% and 0.35% digitonin, respectively. The slopes of the straight lines in both figures gave k_2, which were presented as an Arrhenius plot in Fig. 4c. Thus the activation energy (E_a) for the regeneration of rhodopsin was estimated at 21.8 kcal/mol (0.0086% digitonin) and 22.5 kcal/mol (0.35% digitonin). Other activation parameters, activation enthalpy change ($\Delta H^{\neq}$), activation free energy change ($\Delta G^{\neq}$), and activation entropy change ($\Delta S^{\neq}$), at 25°C and pH 7.0 were calculated by the following equations;

$$\Delta H^{\neq} = E_a - RT$$

$$\Delta G^{\neq} = -RT \ln(k_r h/kT)$$

$$\Delta S^{\neq} = (\Delta H^{\neq} - \Delta G^{\neq})/T$$

R, general gas constant; T, absolute temperature; h, Planck's constant; k,

TABLE I

ACTIVATION PARAMETERS OF THE REGENERATION OF CATTLE RHODOPSIN IN DIGITONIN (25°C, pH 7.0)

	E_a (kcal/mol)	$\Delta H^{\neq}$ (kcal/mol)	$\Delta G^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal/mol per deg)
Below critical micelle concentration (0.0086%)	21.8	21.2	14.3	23.2
Above critical micelle concentration (0.35%)	22.5	21.9	13.2	29.2

Boltzman's constant; k_r, the measured second-order rate constant (k_2) of the regeneration of cattle rhodopsin at 25°C and pH 7.0.
The calculated results are shown in Table I.

Discussion

Regeneration of cattle rhodopsin in digitonin or rod outer segments

The regeneration of rhodopsin in digitonin at 25°C and pH 7.0 can be analyzed using a pseudo first-order treatment in the presence of excess 11-*cis*-retinal (Fig. 1a). At least up to 15 μM of 11-*cis*-retinal the pseudo first-order rate constant (k_{app}) has linearity against the concentration of 11-*cis*-retinal at different concentrations of digitonin (Fig. 1b). The slopes of Fig. 1b give the second-order rate constant (k_2) of the regeneration of rhodopsin. The dependence of k_2 on the concentration of digitonin (Fig. 2a) shows that digitonin remarkably inhibits the regeneration of rhodopsin above the critical micelle concentration. Far below the critical micelle concentration k_2 becomes constant ($7500\ M^{-1} \cdot s^{-1}$). Under this condition endogenous phospholipids rather than digitonin might form micelle with opsin. Thus we believe that the regeneration of rhodopsin below the critical micelle concentration of digitonin is similar to that in rod outer segments.

The regeneration of rhodopsin in rod outer segments was studied in Fig. 3. The second-order rate constant (k_2) in rod outer segments ($5600\ M^{-1} \cdot s^{-1}$) is approx. 25% smaller than that in digitonin below the critical micelle concentration. The difference in k_2 between the digitonin extract and rod outer segments may be due to the relatively large sizes of the opsin aggregates in rod outer segments, which could lower the effective concentration of opsin. Endogenous phospholipids in rod outer segments could also lower the effective concentration of 11-*cis*-retinal by forming Schiff bases [12,13].

Another experiment showed that the addition of 0.35% digitonin in the final concentration did not inhibit regeneration of rhodopsin in rod outer rod outer segments. Thus the idea that the binding of digitonin molecule to a specific binding site in opsin may cause inhibition seems to be inadequate. The change of k_2 at the critical micelle concentration of digitonin (Fig. 2a) suggests that the inhibition of the rhodopsin regeneration by digitonin is caused by the formation of digitonin micelles.

Possible mechanism of the regeneration of rhodopsin and its implications

The competitive inhibition of the regeneration of rhodopsin by β-ionone ($8.71 \cdot 10^{-5}$ M) is almost abolished above the critical micelle concentration of digitonin as shown in Fig. 2b. These results explain the controversy that 11-*cis*-retinol did not inhibit the regeneration of rhodopsin in 2% digitonin (Kropf, A., personal communication), although all-*trans*-retinol did inhibit regeneration at digitonin concentrations below the critical micelle concentration (Matsumoto, H. and Yoshizawa, T., unpublished).

We previously postulated the mechanism of the regeneration of rhodopsin as follows [5]: In the first step, an 11-*cis*-retinal · opsin complex is formed through a non-covalent bond between β-ionone ring of 11-*cis*-retinal and the β-ionone ring-binding site in opsin (Eqn. 2). In the following step which is slow,

the protonated retinylidene Schiff base is irreversibly linked between the aldehyde group of 11-*cis*-retinal and ϵ-NH_2 of L-lysine on the chromophore site of opsin (Eqn. 3).

$$11\text{-}cis\text{-retinal} + \text{opsin} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} 11\text{-}cis\text{-retinal} \cdot \text{opsin complex} \tag{2}$$

$$11\text{-}cis\text{-retinal} \cdot \text{opsin complex} \xrightarrow{k_{+2}} \text{rhodopsin} \tag{3}$$

It should be noted that in Eqn. 3 the formation of Schiff base of rhodopsin is irreversible, which could be caused by protection from solvent by the opsin environment.

If the equilibrium in Eqn. 2 is very fast in comparison with the succeeding reaction (Eqn. 3), which seems true because Eqn. 2 is a step of non-covalent bond formation and Eqn. 3 is that of covalent bond formation, the dissociation constant of the 11-*cis*-retinal · opsin complex, K_m, can be expressed as follows:

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \cong \frac{k_{-1}}{k_{+1}} \,. \tag{4}$$

No one knows the value of K_m represented by Eqn. 4, but it is not an irrelevant assumption if we use the inhibition constant of the regeneration of rhodopsin by β-ionone or all-*trans*-retinol for K_m. Thus supposing that K_m is 10^{-4} M, we can calculate the concentration of 11-*cis*-retinal · opsin complex at least ten times smaller than that of 11-*cis*-retinal and free opsin under the experimental conditions. As the reaction proceeds under the pseudo first-order condition of 11-*cis*-retinal excess, the concentrations of 11-*cis*-retinal · opsin complex and rhodopsin are negligible compared with free 11-*cis*-retinal and opsin. Then the apparent pseudo first-order rate constant (k_{app}) can be represented by the following equation,

$$k_{app} = \frac{[11\text{-}cis\text{-retinal}]_0}{[11\text{-}cis\text{-retinal}]_0 + K_m} \cdot k_{+2} \,, \tag{5}$$

where $[11\text{-}cis\text{-retinal}]_0$ is the initial concentration of 11-*cis*-retinal. Eqn. 5 indicates that k_{app} increases up to a saturation level as 11-*cis*-retinal increases. As far as we examined, k_{app} did not saturate at least up to 30 μM of 11-*cis*-retinal at 25°C and pH 7.0 (data not shown). As 11-*cis*-retinal is much smaller than K_m, which could be true because K_m is similar to the inhibition constant of β-ionone (approx. 100 μM), Eqn. 5 can be written approximately as

$$k_{app} = \frac{k_{+2}}{k_m} \cdot [11\text{-}cis\ \text{retinal}]_0 \,. \tag{6}$$

The fact that the inhibition of the regeneration of rhodopsin by β-ionone diminishes above the critical micelle concentration of digitonin suggests that when digitonin forms micelles the dissociation constant of the 11-*cis*-retinal · opsin complex (K_m) and similarly the dissociation constant of the β-ionone · opsin complex (K_I) increase 150 times larger (Fig. 2a) than that when digitonin does not form micelles. This implies that the rate-determining step of the regeneration of rhodopsin does not change above and below the critical micelle concentration of digitonin. The fact that the activation energies of the

regeneration of rhodopsin have almost a constant value, approx. 22 kcal/mol (Fig. 4c), can be explained by the increase of the dissociation constant, assuming that K_m has the similar temperature dependence under both conditions.

Another possible explanation that digitonin micelles may trap 11-*cis*-retinal and lower the effective concentration of it compared to that below the critical micelle concentration cannot be excluded. But the fact that the addition of digitonin to rod outer segments above the critical micelle concentration did not change the kinetics as mentioned above seems difficult to be explained by it.

Activation parameters of the regeneration of rhodopsin are summarized in Table I. In consideration of these parameters, especially of the large positive values of the activation entropy changes ($\Delta S^{\neq}$) under both conditions, it should be noted that, according to the above-mentioned mechanism, the second-order rate constant (k_2) can be written as

$$k_2 = \frac{k_{app}}{[11\text{-}cis\text{-retinal}]_0} = \frac{k_{+2}}{K_m}. \quad (7)$$

Therefore the activation parameters calculated from k_2 (Table I) do not represent the true activation parameters in the sense that they contain temperature dependence both of the 11-*cis*-retinal · opsin complex formation (Eqn. 2) and of the protonated Schiff base formation (Eqn. 3). For example the activation energies (E_a) shown in Table I should be apparent and contain: (a) the heat of formation of the 11-*cis*-retinal · opsin complex, and (b) the energy of activation of this complex (see ref. 14). We tried to measure k_{+2} and K_m in Eqn. 5 separately, but in vain, because k_{app} is too fast to measure when [11-*cis*-retinal]$_0$ is close to K_m (higher than 30 μM) by the usual method (Method I or Method II). Thus further kinetic studies are needed to explain the unusually large positive values of $\Delta S^{\neq}$ and other activation parameters.

Significance of the rate constant of the regeneration of rhodopsin to the process of dark adaptation

Recently Henselman and Cusanovich [15] measured the regeneration of cattle rhodopsin in rod outer segments. Their results were different from ours even though conditions were similar (25°C and pH 7.0). Thus they obtained the value of 690 $M^{-1} \cdot s^{-1}$ as the second-order rate constant for the regeneration of rhodopsin under pseudo first-order condition of 11-*cis*-retinal excess when 11-*cis*-retinal was 20 μM, while in the present work (Fig. 3b) k_2 is 5600 $M^{-1} \cdot s^{-1}$. This discrepancy must be attributed to the fact that they used the increase of absorbance at 500 nm to measure the amount of regenerated rhodopsin. Since random retinylidene Schiff bases have some absorbance at 500 nm, one cannot estimate exactly the amount of the regenerated rhodopsin by measuring the increase of the absorbance at 500 nm. Although the contribution of random Schiff bases to the 500 nm absorbance is relatively small (10—15% of the total absorbance at 500 nm), it seriously effects the kinetics. When we measure the increase of 500 nm absorbance for the regeneration of rhodopsin in rod outer segments the pseudo first-order plot becomes biphasic. We confirmed that the slow phase represents the formation of random Schiff bases and the fast phase

represents the formation of rhodopsin by comparing the results obtained from Method I and Method II.

The rate of regeneration of rhodopsin in vivo, which is directly related to the process of dark adaptation, is similar to the second-order rate constant (k_2) below the critical micelle concentration of digitonin (Fig. 2a) or in rod outer segments (Fig. 3b). Rushton [16] measured the regeneration of human rhodopsin in vivo, and found that the regeneration always occurred along an exponential curve of approx. 4 min half-time ($t_{1/2}$) when the bleached fractions of rhodopsin varied from 25 to 100%. If we assume that the regeneration of cattle rhodopsin in vivo also occurs along an exponential curve, the nature of the dark adaptation can roughly be characterized. Namely, if one assumes that $t_{1/2}$ is 4 min in the living eye of cattle, the concentration of 11-*cis*-retinal in situ can be calculated using Eqn. 1;

$$[\text{11-}cis\text{-retinal}]_{\text{in situ}} = k_{\text{app}}/k_2$$

$$= \frac{(0.693/t_{1/2})}{k_2}$$

$$= 2.9 \cdot 10^{-7}\ \text{M},$$

where k_2 is assumed to be approx. $10^4\ \text{M}^{-1} \cdot \text{s}^{-1}$ at physiological temperature (Fig. 4c). In this calculation one should notice that Eqn. 1 is valid not only when 11-*cis*-retinal is excess over opsin but also when 11-*cis*-retinal is constant under an opsin excess condition.

The concentration of rhodopsin in rod outer segments is supposed to be in the order of 10^{-3} M in a frog retina [17]. These data, even though collected from different species of animals, suggest that the regeneration of rhodopsin in vivo could be performed under pseudo first-order condition of opsin excess at a constant concentration of 11-*cis*-retinal (approx. 10^{-7} M).

The mechanism of the constancy in the concentration of 11-*cis*-retinal is unknown, but could be an existence of a pool of a precursor of 11-*cis*-retinal. One possible explanation is that the precursor is an 11-*cis*-retinol which is oxidized into 11-*cis*-retinal by retinol dehydrogenase in rod outer segments [18]. The concentration of 11-*cis*-retinol in rod outer segments is approx. 10^{-4} M, because the content of 11-*cis*-retinol in rod outer segments of a frog retina is approx. 2% of rhodopsin [19] and the concentration of rhodopsin is approx. 10^{-3} M [17]. Meanwhile the concentration of 11-*cis*-retinal in situ was calculated to be approx. 10^{-7} M by us. Therefore 11-*cis*-retinol in rod outer segments can be a pool in supplying 11-*cis*-retinal at a constant concentration of 10^{-7} M.

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