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TWO FORMS OF INTERMEDIATES OF FROG RHODOPSIN IN ROD OUTER SEGMENTS

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Using frog rod outer segments, we measured changes of the absorption spectrum during the conversion of rhodopsin to a photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin by irradiation with blue light (440 nm) at −190°C and during the reversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin by irradiation with red light (718 nm) at −190°C. The reaction kinetics was expressed by one exponential in the former case and by two exponentials in the latter. These results suggest that rhodopsin is composed of a single molecular species, while bathorhodopsin is composed of two kinds of molecular species designated as batho$_1$-rhodopsin and batho$_2$-rhodopsin. On warming the two forms of bathorhodopsin, each bathorhodopsin converted to its own lumirhodopsin, metarhodopsin I and finally a free all-*trans*-retinal plus opsin. The absorption spectra of the two forms of bathorhodopsin, lumirhodopsin and metarhodopsin I were measured at −190°C. We infer that a rhodopsin molecule in the excited state relaxes to either batho$_1$-rhodopsin or batho$_2$-rhodopsin, and then converts to its own intermediates through one of the two parallel pathways.

Introduction

On absorption of light, rhodopsin decomposes via a series of intermediates to the final photoproduct, all-*trans*-retinal plus opsin. The primary photoproduct of rhodopsin, which we now call bathorhodopsin, was first observed by Yoshizawa and Kito [1], who irradiated rhodopsin at liquid nitrogen temperatures.

According to flash-photolysis experiments, the kinetics in the thermal conversion of bathorhodopsin to lumirhodopsin were fitted with three exponentials [2,3]. Recent kinetic studies on conversion of lumirhodopsin to metarhodopsin I and of metarhodopsin I to metarhodopsin II showed that lumirhodopsin is composed of two kinds of isochromic components, each of which decays to its own metarhodopsin I [4]. Thus, it seems likely that all the thermal intermediates may be in multiple forms. Our recent studies on bovine rhodopsin and isorhodopsin showed that they are composed of a single molecular species at 77 K [5,6], whereas bovine hypsorhodopsin and bathorhodopsin are composed of two kinds of molecular species, which differ in their absorption spectrum. The difference in absorption spectrum between two forms of hypsorhodopsin or bathorhodopsin is probably due to the difference in interaction between the retinylidene chromophore and the opsin [5,6].

The purpose of the present paper is to confirm the existence of two forms of bathorhodopsin, lumirhodopsin and metarhodopsin I in the frog rod outer segment system and to describe their absorption spectra and thermal stabilities.

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Materials and Methods

Preparation of rod outer segments. All the procedures were performed under dim red light. Rod outer segments were isolated from bullfrog (*Rana catesbeiana*) retinas by a sucrose (34%) flotation method [5]. The crude rod outer segments thus obtained were purified by a slight modification [5,7] of the method of Kimura [8] as follows: The rod outer segments were suspended in a sucrose solution (density 1.10 g/ml) containing 2 mM $MgCl_2$ and 65 mM NaCl in 10 mM Hepes buffer (pH 6.8). After homogenization, the rod outer segment suspension was put on the top of a discontinuous sucrose gradient solution (densities 1.11, 1.13 and 1.15 g/ml) buffered with 10 mM Hepes (pH 6.8), and then centrifuged at $63\,600 \times g$ for 1 h. The rod outer segments came together at the interface between 1.11 and 1.13 g/ml sucrose layers. After collection they were washed with the buffer solution mixed with glycerol and neutralized hydroxylamine at final concentrations of 75% and 0.1 M, respectively. This rod outer segment preparation was used as a sample for low-temperature spectrophotometry.

Spectrophotometry. Absorption spectra of rhodopsin and its photoproducts in rod outer segment preparation were recorded at a constant temperature from 300 to 77 K with a specially constructed glass cryostat [9] attached to an automatic recording spectrophotometer (Hitachi 323 type). The cryostat has an optical cell consisting of a silicone-rubber ring (approx. 1 mm thickness), a front quartz and a back opal glass. The opal glass was placed on the reference side. The temperature of the sample was monitored with a copper-constantan thermocouple fixed in the sample holder. The light source for irradiation of the sample was a xenon lamp (2 kW, Ushio Co., Japan). The wavelength of irradiation light was selected by inserting a cutoff filter of a pair of cutoff and interference filters (Toshiba, Japan) between the sample and the light source.

Results

The kinetic analysis of photoconversion of rhodopsin at 20°C

In order to examine whether or not frog rhodopsin in rod outer segments is in multiple forms, the kinetics of photobleaching of rhodopsin were measured by irradiation with orange light at wavelengths longer than 560 nm at 20°C. The relative absorbance changes at various wavelengths were plotted against the exposure time on a semilogarithmic scale. The following formula, $(A_t - A_\infty)/(A_0 - A_\infty)$, gives the relative absorbance changes, where A_0, A_t and A_∞ denote the absorbances at a particular wavelength at times zero, t and infinity, respectively. The kinetics thus obtained could be expressed by one exponential at all wavelengths except those in the wavelength region near the isosbestic point (415 nm) between rhodopsin and all-*trans*-retinal oxime. Thus, we concluded that the rhodopsin is composed of a single molecular species or two isochromic forms which are in thermal equilibrium.

Photoconversion of rhodopsin to bathorhodopsin

On cooling from room temperature to 77 K with liquid nitrogen, the λ_{max} of frog rhodopsin in rod outer segment preparation moved from 502 to 515 nm.

Irradiation of the rhodopsin at −190°C with blue light of 440 nm shifted the spectrum to longer wavelength, indicating formation of bathorhodopsin (Fig. 1a). Prolonged irradiation yielded a photosteady-state mixture (curve 6 in Fig. 1a) composed of 58% bathorhodopsin, 21% rhodopsin and 21% isorhodopsin. These percentages were calculated according to a slightly modification [10] of the method of Yoshizawa and Wald [11]. The kinetics of photoconversion of rhodopsin to bathorhodopsin were fitted with one exponential, as shown in the inset of Fig. 1a. Thus, we confirmed that the rhodopsin is composed of a single molecular species, like bovine rhodopsin and isorhodopsin [5].

Photoconversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin

The photosteady-state mixture containing bathorhodopsin (58%) was irradiated at −190°C with red light (718 nm) which can be absorbed only by bathorhodopsin. The spectrum shifted to shorter wavelength, indicating photoconversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin (Fig. 1b). However, the curve-inter-

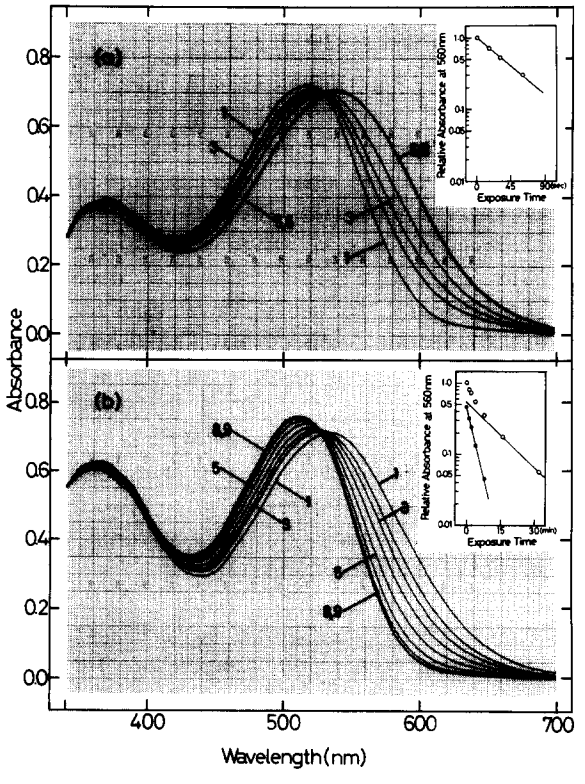

Fig. 1. Interconversion among rhodopsin, isorhodopsin and bathorhodopsin by light at −190°C. (a) Photoconversion of rhodopsin to bathorhodopsin. (Curve 1) Absorption spectrum of rhodopsin in rod outer segments at −190°C. (Curves 2–6) Products of irradiation with blue light (440 nm) for successive periods of 15, 15, 30, 60 and 120 s. (Inset) Kinetics of photoconversion of rhodopsin to bathorhodopsin. (b) Photoconversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin. (Curve 1) Photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin (identical with curve 6 in a). (Curves 2–9) Products of irradiation of the mixture with red light (718 nm) for successive periods of 1, 1, 2, 4, 8, 16, 32 and 64 min. (Inset) Kinetics of photoconversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin.

section point moved from 534 to 522 nm with increase in exposure time. The kinetics of the photoconversion from bathorhodopsin to a mixture of rhodopsin and isorhodopsin are shown in the inset of Fig. 1b (open circles). For a long period of irradiation the kinetics are expressed by a straight line due to photoconversion of a slow component. Each value on the kinetic curve was subtracted from the corresponding value on the straight line extrapolated. Replotting the values thus obtained (closed circles) gave another straight line due to photoconversion of a fast component.

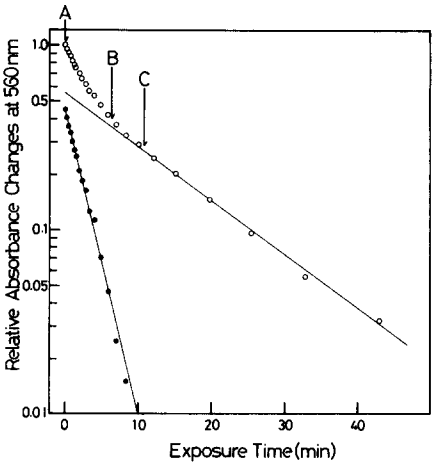

Fig. 2. Kinetics of photoconversion of bathorhodopsin to a mixture of rhodopsin and isorhodopsin. (Point A) Before irradiation. (Point B) Bathorhodopsin was reduced to 40% of the initial amount for conversion to a mixture of rhodopsin and isorhodopsin by irradiation with red light (718 nm). (Point C) Bathorhodopsin was reduced to 28% of the initial amount for conversion to a mixture of rhodopsin and isorhodopsin by red light irradiation.

Fig. 2 shows an example of more detailed analysis of the kinetics plotted at 560 nm, which were expressed by two exponentials. The kinetics at other wavelengths were also expressed by two exponentials. Thus, we concluded that bathorhodopsin is composed of two components; the fast component is tentatively termed batho$_1$-rhodopsin and the slow component batho$_2$-rhodopsin.

Thermal conversion of bathorhodopsin via lumirhodopsin to metarhodopsin I

The familiar photosteady-state mixture composed of bathorhodopsin (58%), rhodopsin (21%) and isorhodopsin (21%) was warmed to −160°C. Almost no spectral change was observed, indicating that bathorhodopsin was stable below −160°C (curves 1–4 in Fig. 3a). Above −160°C the spectrum shifted to shorter wavelengths, forming an isosbestic point around 535 nm (curves 4–11 in Fig. 3b). Since curve 10 coincides with curve 11, it seems reasonable to assume that all the bathorhodopsin in the preparation may be converted to lumirhodopsin. On further warming above −80°C, the absorption spectrum shifted to shorter wavelengths with an isosbestic point at 500 nm (curves 11–16 in Fig. 3c), owing to conversion

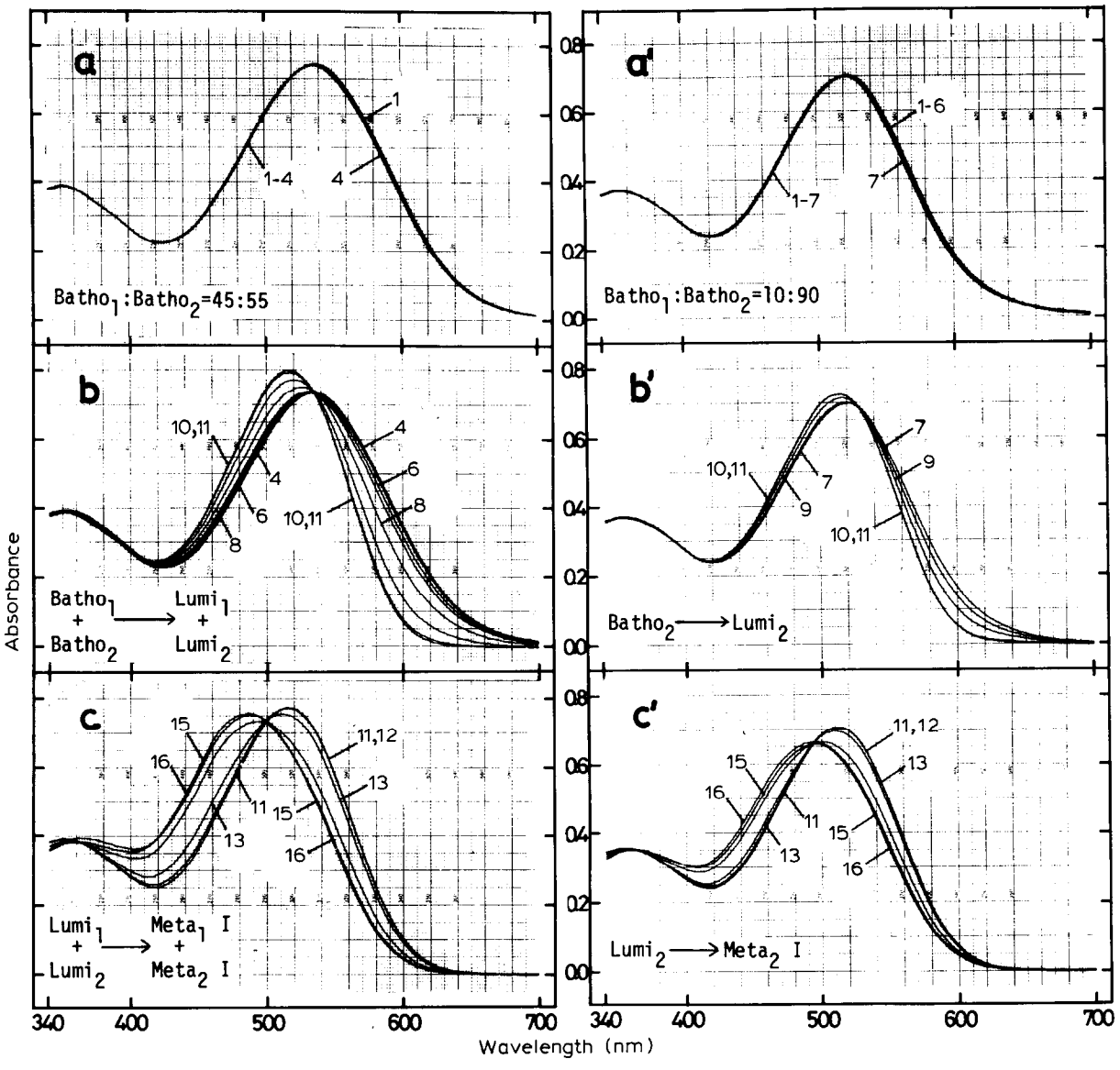

Fig. 3. Thermal conversion of two forms of bathorhodopsin. (a) Spectral change of a photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin (batho$_1$/batho$_2$ = 45 : 55) on warming from −190 to −160°C. The photosteady-state mixture (curve 1) (corresponding to curve 6 in Fig. 1a) was warmed to −180 (curve 2), −170 (curve 3) and −160°C (curve 4). Each time the preparation was recooled to −190°C for measuring the spectrum. (b) Spectral change on thermal conversion of bathorhodopsin (batho$_1$- and batho$_2$-rhodopsins) to lumirhodopsin (lumi$_1$- and lumi$_2$-rhodopsins). The photosteady-state mixture (curve 1) was warmed to −160 (curve 4), −150 (curve 5), −140 (curve 6), −130 (curve 7), −120 (curve 8), −110 (curve 9), −100 (curve 10) and −90°C (curve 11). Each time the preparation was recooled to −190°C for measuring the spectrum. (c) Spectral change on conversion of lumirhodopsin (lumi$_1$- and lumi$_2$-rhodopsins) to metarhodopsin I (meta$_1$- and meta$_2$-rhodopsins I). The photosteady-state mixture (curve 1) was warmed to −90 (curve 11), −80 (curve 12), −70 (curve 13), −60 (curve 14), −50 (curve 15) and −40°C (curve 16). Each time the preparation was recooled to −90°C for measuring the spectrum. (a') Spectral change of mainly batho$_2$-rhodopsin on warming from −190 to −130°C. A mixture composed of rhodopsin, isorhodopsin and batho$_2$-rhodopsin with a small amount of batho$_1$-rhodopsin (batho$_1$/batho$_2$ = 10 : 90) (curve 1) was warmed to −180 (curve 2), −170 (curve 3), −160 (curve 4), −150 (curve 5), −140 (curve 6) and −130°C (curve 7). Each time, the preparation was recooled to −190°C for measuring the spectrum. (b') Spectral change on conversion of mainly batho$_2$-rhodopsin to lumi$_2$-rhodopsin. The mixture (curve 1) was warmed to −130 (curve 7), −120 (curve 8), −110 (curve 9), −100 (curve 10) and −90°C (curve 11). Each time the preparation was recooled to −190°C for measuring the spectrum. (c') Spectral change on conversion of lumi$_2$-rhodopsin to meta$_2$-rhodopsin I. The mixture (curve 1) was warmed to −90 (curve 11), −80 (curve 12), −70 (curve 13), −60 (curve 14), −50 (curve 15) and −40°C (curve 16). Each time

of lumirhodopsin to metarhodopsin I. On warming above −40°C, metarhodopsin I converted to all-*trans*-retinal oxime plus opsin.

Formation of lumi$_2$-rhodopsin and meta$_2$-rhodopsin I

A preparation containing batho$_2$-rhodopsin with a small amount of batho$_1$-rhodopsin was prepared in the following way: Since bathorhodopsin (58%) in the familiar photosteady-state mixture is a mixture of batho$_1$- and batho$_2$-rhodopsins (Fig. 2), the batho$_1$-rhodopsin was removed from the preparation by means of converting 60% of the initial amount of the bathorhodopsin with red light of wavelength 718 nm (point B in Fig. 2). This preparation contains bathorhodopsin composed of 10% batho$_1$-rhodopsin and 90% batho$_2$-rhodopsin as described in a later section.

On warming this preparation (curve 1 in Fig. 3a′) to −140°C, the spectrum changed only a little, indicating that batho$_2$-rhodopsin was stable below −140°C (curves 1–6 in Fig. 3a′). Above −140°C the spectrum shifted to shorter wavelengths with an isosbestic point at 528 nm (curves 7–11 in Fig. 3b′), owing to the thermal convresion of batho$_2$-rhodopsin to lumi$_2$-rhodopsin. On further warming the preparation (curve 11 in Fig. 3b′) above −80°C, the spectrum showed another shift to shorter wavelengths, forming an isosbestic point around 495 nm. This indicates that lumi$_2$-rhodopsin was converted to meta$_2$-rhodopsin I. Above −40°C, meta$_2$-rhodopsin I was converted to all-*trans*-retinal oxime and opsin.

Absorption spectra of two forms of intermediates

(i) Mixtures of two forms of intermediates. When the familiar photosteady-state mixture (curve 1 in Fig. 4a) was warmed from −190 to −90°C, the absorption spectrum shifted to shorter wavelengths (curve 2). Since the rhodopsin and isorhodopsin in the mixture remain unchanged, the spectral shift should be due to conversion of bathorhodopsin in the mixture to lumirhodopsin. Further warming to −40°C caused another spectral shift to shorter wavelengths (curve 3), owing to conversion of the lumirhodopsin to metarhodopsin I. Thus, the spectrum (curve 3) which was measured after recooling to −190°C shows a mixture of metarhodopsin I, rhodopsin and isorhodopsin.

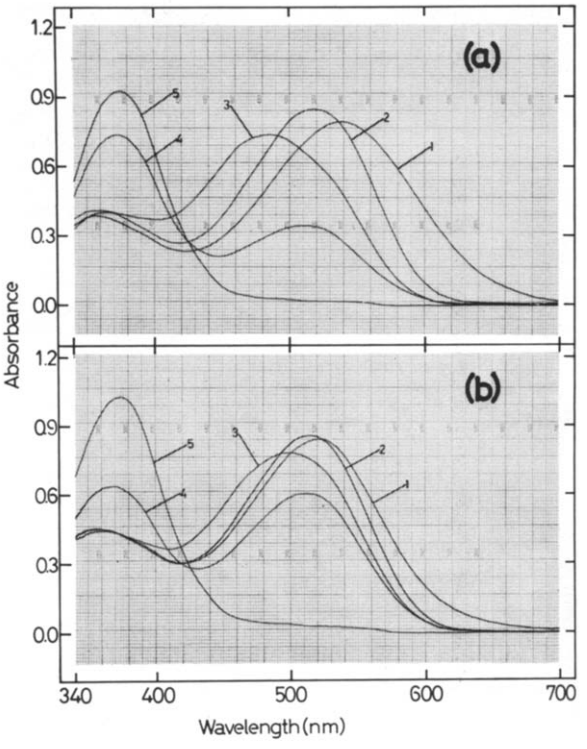

Fig. 4. Two sets of absorption spectra for calculating the absorption spectra of two forms of intermediates at −190°C. (a) A photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin (batho$_1$/batho$_2$ = 45:55) was prepared by irradiation of the rod outer segment preparation with blue light (440 nm) at −190°C (curve 1). The mixture was warmed to −90 (curve 2), −40 (curve 3) and then 20°C (curve 3) for formation of lumirhodopsin, metarhodopsin I and all-*trans*-retinal oxime plus opsin, respectively, and each time recooled to 190°C for measurement of the spectra. After complete bleaching at room temperature with orange light ($\lambda > 520$ nm), the preparation was recooled to −190°C (curve 5). The spectrum represents all-*trans*-retinal oxime plus opsin. (b) Curve 1 represents a mixture of rhodopsin, isorhodopsin and bathorhodopsin (batho$_1$/batho$_2$ = 10:90) at −190°C, which was prepared by irradiating the photosteady-state mixture with red light (718 nm) at −190°C. (Curves 2–5) Corresponding to curves 2–5 in a.

Now the preparation was warmed to 20°C. It was converted to a mixture composed of all-*trans*-retinal oxime plus opsin, rhodopsin and isorhodopsin. The difference in absorbance at 508 nm (where an isosbestic point between rhodopsin and isorhodopsin at room temperature is located) between this mixture and the original rhodopsin at 20°C gives a measure of the amount of an inter-

mediate (bathorhodopsin, lumirhodopsin or metarhodopsin I) in the preparation. The amount of each intermediate in the preparation was calculated to be 58% of the original rhodopsin. If curve 4, which is a spectrum of the mixture composed of all-*trans*-retinal oxime plus opsin, rhodopsin and isorhodopsin at $-190°C$, is subtracted from curve 2 (the spectrum of the mixture of bathorhodopsin, rhodopsin and isorhodopsin), the difference should give a difference spectrum between bathorhodopsin and all-*trans*-retinal oxime plus opsin.

Recooling the preparation to $-190°C$, however, caused different degrees of intensification of the spectrum each time, due to formation of cracks in the preparation, which induce multiple reflection of the measuring light [12]. Therefore, the extent of intensification by the recooling was corrected by comparing the absorbance at 508 nm at room temperature with that at 513 nm at $-190°C$, where each isosbestic point between rhodopsin and isorhodopsin is located.

Thus, the difference spectrum between bathorhodopsin and all-*trans*-retinal oxime plus opsin was accurately calculated. The addition of the difference spectrum to the spectrum of all-*trans*-retinal oxime plus opsin (58% of curve 5) gave a spectrum of bathorhodopsin. The λ_{max} of bathorhodopsin was at 562 nm (± 2 nm), which is very close to a value (λ_{max} 564 nm) previously reported [13]. In the same manner, absorption spectra of lumirhodopsin and metarhodopsin I were calculated from curves 2 and 3, respectively. The λ_{max} values of lumirhodopsin and metarhodopsin I derived from bathorhodopsin by warming were at 526 nm (± 2 nm) and 470 nm (± 2 nm) (average of five experiments). We shall tentatively denote bathorhodopsin, lumirhodopsin and metarhodopsin I by $Batho_{440}$, $Lumi_{440}$ and $Meta\ I_{440}$, respectively.

(ii) Two forms of intermediates. As seen in Fig. 2, irradiation of the familiar photosteady-state mixture with 718 nm light for 11 min (point C) reduced the bathorhodopsin to 28% of its initial amount. This preparation consisted of $batho_2$-rhodopsin with a small amount of $batho_1$-rhodopsin. On warming to 20°C in the dark, $batho_2$-rhodopsin in the preparation was bleached to all-*trans*-retinal oxime plus opsin. A difference in absorbance at 508 nm between this preparation and the original preparation at room temperature represented the amount of $batho_2$-rhodopsin (16 ± 1%) in the preparation. After recooling the preparation, the absorption spectrum was measured. After the correction for the effect of recooling was made as described in the previous section, the spectrum was subtracted from that of the preparation consisting of rhodopsin, isorhodopsin and $batho_2$-rhodopsin. Thus, a difference spectrum between $batho_2$-rhodopsin and all-*trans*-retinal oxime plus opsin was obtained. The addition of the difference spectrum to the spectrum of all-*trans*-retinal oxime plus opsin (curve 5×16%) gave the spectrum of $batho_2$-rhodopsin. The λ_{max} of $batho_2$-rhodopsin was 548 nm (± 3 nm). In the same manner, the λ_{max} values of the absorption spectra of $lumi_2$-rhodopsin and $meta_2$-rhodopsin I were at 522 nm (± 2 nm) and 467 nm (± 3 nm), respectively.

As mentioned above, the amount of $batho_2$-rhodopsin was so small (16%) that the intensity and shape of the spectrum were greatly affected by the degrees of cracks formed in the preparation. Then, the following method was used for calculation of the spectrum of two forms of intermediates.

First, the ratio of $batho_1$-rhodopsin to $batho_2$-rhodopsin in $Batho_{440}$ was calculated as follows: Since irradiation of $Batho_{440}$ for 11 min with red light (718 nm) brought the amount of $batho_2$-rhodopsin to 16% of the original rhodopsin, which corresponds to a decrease in $batho_2$-rhodopsin from 55 to 28% according to the upper straight line in Fig. 2, the amount of $batho_2$-rhodopsin before irradiation was calculated to be $16\% \times (55/28) = 32\%$. Since the amount of $Batho_{440}$ in the photosteady-state mixture had been estimated to be 58%, the amount of $batho_2$-rhodopsin in $Batho_{440}$ was calculated to be $(32/58) \times 100\% = 55\%$. The reason why this value (55%) is in good agreement with that shown by the upper straight line in Fig. 2 is that an isosbestic point between $batho_1$-rhodopsin and $batho_2$-rhodopsin is located at 560 nm, where the relative absorbance change is plotted in Fig. 2. Thus, it is concluded that $Batho_{440}$ is composed of 45% $batho_1$-rhodopsin and 55% $batho_2$-rhodopsin.

Secondly, the preparation at point B on the kinetic curve shown in Fig. 2 was used for the

calculation of absorption spectra of the intermediates. In this preparation, the amount of bathorhodopsin decreased to 40% of $Batho_{440}$, and that of $batho_1$-rhodopsin decreased to 4% of $Batho_{440}$ according to the lower straight line. The ratio of $batho_1$-rhodopsin to $batho_2$-rhodopsin in this preparation was calculated to be 10 : 90. From the set of spectra shown in Fig. 4b, the absorption spectra of bathorhodopsin, lumirhodopsin and metarhodopsin I were calculated using the procedure described in section i. The absorption maxima of bathorhodopsin, lumirhodopsin and metarhodopsin I were at 552, 523 and 468 nm, respectively, in the averages of four experiments.

Finally, the two sets of absorption spectra of intermediates, which differ in the ratio of the two forms of intermediates, were used for the calculation of each absorption spectrum in the two forms of intermediates. Absorption spectra of $batho_1$-rhodopsin [$\varepsilon_{B_1}(\lambda)$] and $batho_2$-rhodopsin [$\varepsilon_{B_2}(\lambda)$] were calculated from the following equations.

$$45\%\,\varepsilon_{B_1}(\lambda)+55\%\,\varepsilon_{B_2}(\lambda)=\varepsilon_B^a(\lambda)$$

$$10\%\,\varepsilon_{B_1}(\lambda)+90\%\,\varepsilon_{B_2}(\lambda)=\varepsilon_B^b(\lambda)$$

where $\varepsilon_B^a(\lambda)$ or $\varepsilon_B^b(\lambda)$ is the extinction coefficient of the mixture of two bathorhodopsins relative to rhodopsin. The absorption spectra of $batho_1$- and $batho_2$-rhodopsins are shown by solid circles in

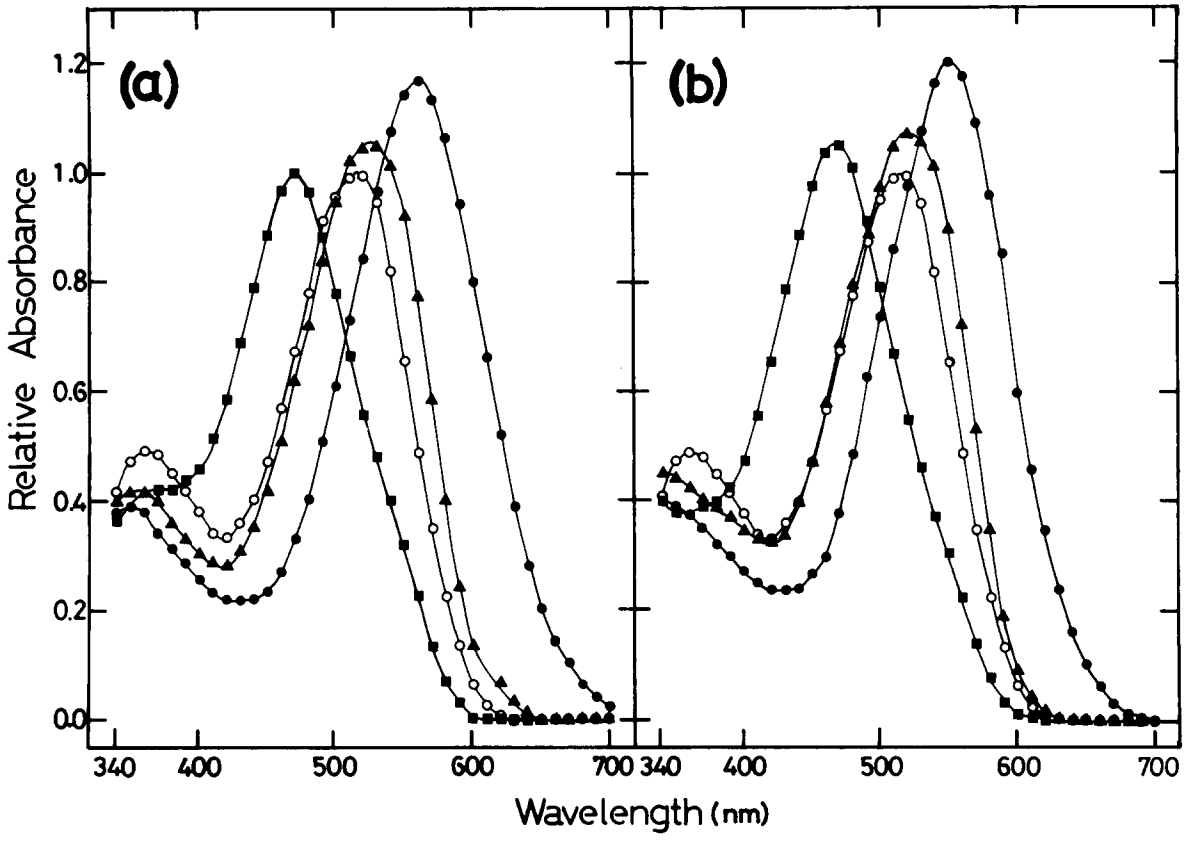

Fig. 5. Calculated absorption spectra of two forms of intermediates at −190°C. (a) $Intermediate_1$. (○———○) Absorption spectrum of frog rhodopsin at −190°C. (●———●) $Batho_1$-rhodopsin (λ_{max} 577 nm). (▲———▲) $Lumi_1$-rhodopsin (λ_{max} 533 nm). (■———■) $Meta_1$-rhodopsin I (λ_{max} 472 nm). The maximum absorbances of $batho_1$-rhodopsin, $lumi_1$-rhodopsin and $meta_1$-rhodopsin I are 1.23-, 1.03- and 0.92-times that of rhodopsin, respectively. (b) $Intermediate_2$. (○———○) Absorption spectrum of frog rhodopsin at −190°C. (●———●) $Batho_2$-rhodopsin (λ_{max} 548 nm). (▲———▲) $Lumi_2$-rhodopsin (λ_{max} 522 nm). (■———■) $Meta_2$-rhodopsin I (λ_{max} 467 nm). The maximum absorbances of $batho_2$-rhodopsin, $lumi_2$-rhodopsin, and $meta_2$-rhodopsin I are 1.21-, 1.08- and 1.06-times that of rhodopsin, respectively.

Fig. 5a and b, respectively. Similar calculations were performed for two forms of lumi- and metarhodopsins (Fig. 5).

Discussion

The present experiment of the bleaching process of frog rhodopsin is summarized in Fig. 6. According to flash-photolysis experiments on bovine and frog rhodopsins at room temperature and low temperature [2,3], lumirhodopsin and metarhodopsin I have each been shown to be composed of three molecular species, presumably having the same absorption spectra. It was also shown that rhodopsin itself is composed of three molecular species [14].

The present results show that each intermediate is composed of two molecular species which differ in their absorption spectrum and thermostability, whereas rhodopsin itself is spectroscopically composed of a single molecular species. In a previous paper, we reported that bovine hypsorhodopsin and bathorhodopsin are composed of two molecular species, which differ in their absorption spectrum [4,5]. Recently, we found two forms of the first bathochromic photoproduct (photointermediate) in the 5,6-dihydrorhodopsin system, which is converted into the hypso intermediate by warming [15].

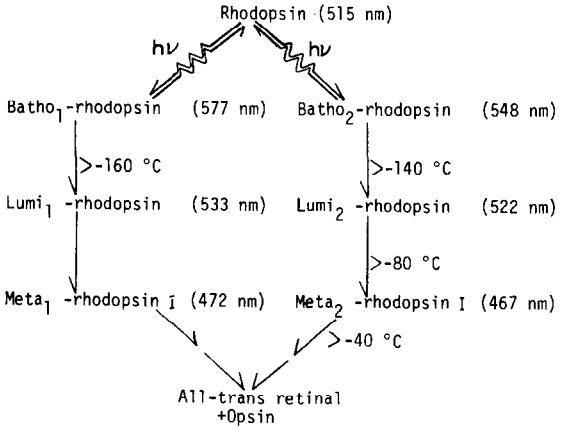

Fig. 6. Diagram showing two parallel pathways of bleaching of rhodopsin. The λ_{max} values of rhodopsin and intermediates (shown in parentheses) were measured at liquid nitrogen temperatures. The transition temperatures are shown on the right-hand side of the arrows, but those from lumi$_1$-rhodopsin to meta$_1$-rhodopsin I and from meta$_1$-rhodopsin I to all-*trans*-retinal plus opsin have not yet been characterized.

All the experiments we had performed on two forms of intermediates of rhodopsin showed that each intermediate is composed of two molecular species, while rhodopsin and isorhodopsin (9-*cis* form) are both a single molecular species as inferred from their spectrum and photosensitivity. These results may suggest that the selection between two bleaching pathways should be made in the first step of the primary photochemical event [5,6]. Since isomerization of the double bond (C_9–C_{10} for isorhodopsin and C_{11}–C_{12} for rhodopsin) may occur via rotation in two directions, i.e., clockwise and counterclockwise, an excited rhodopsin molecule may relax through either of two parallel pathways.

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