Absorption spectrum of cattle hypsorhodopsin

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On irradiation at liquid helium temperatures, rhodopsin is converted into hypsorhodopsin which decays to bathorhodopsin above 23 K. The absorption spectrum of cattle hypsorhodopsin (λ_{max} = 435 nm) is found to include a new sideband around 540 nm. This sideband may be due to $\pi^* \longleftarrow \pi$ transition to $^1A_g^-$ like state, which is made partially allowed by distortion of polyene chain of the retinylidene chromophore.

Photoconversion *Rhodopsin* *Liquid helium temperature* *Hypsorhodopsin*

1. INTRODUCTION

Rhodopsin is a photoreceptive pigment for vision. On absorption of light, rhodopsin is bleached through a series of intermediates characterized by their absorption spectra and thermostabilities. When rhodopsin is irradiated at liquid helium temperatures (4–9 K), hypsorhodopsin as well as bathorhodopsin is produced [1,2]. Bathorhodopsin is the same intermediate as that previously called prelumirhodopsin which is stable at liquid nitrogen temperatures (77–83 K) [3]. It has been shown that on warming to 77 K, hypsorhodopsin decays to bathorhodopsin, leading to the conclusion that hypsorhodopsin is a precursor of bathorhodopsin [1,2]. However, the finding that the quantum yield of formation of hypsorhodopsin at 4–9 K is low in comparison with that of bathorhodopsin [1,2,4–8], has led to the suggestion that hypsorhodopsin might be an intermediate which is not on the main pathway of light-activated reaction [1,2,6,8,9]. Apart from the problem as to the location of hypsorhodopsin in the primary process of rhodopsin cycle, there is still another incomprehensible phenomenon in behaviour of rhodopsin and its photoproduct at 4–9 K; in a variety of animal rhodopsins the content of hypsorhodopsin in any photosteady state formed by irradiation of rhodopsin or isorhodopsin at 4–9 K appears never to exceed the limit of 65% [1,2,4–7]. Our studies of absorption spectra of vertebrate hypsorhodopsins, have revealed the indication of a sideband around 540 nm. However, insufficient data existed to determine whether the sideband is intrinsic to hypsorhodopsin or is a contamination of bathorhodopsin (cattle: 548 nm at 4 K) in the preparation. An attempt is here made to resolve this question.

2. MATERIALS AND METHODS

All the procedures were performed under dim red light. Rod outer segments were isolated from cattle retinas according to conventional sucrose flotation method [2]. Rhodopsin was extracted from the rod outer segments with 2% digitonin (66 mM phosphate buffer, pH 6.4). The extract was mixed with hydroxylamine and glycerol at final concentrations of 50 mM and 75%, respectively. This mixture was used as a sample.

Low temperature spectrophotometry was done using two kinds of glass cryostats [1,7], each of which was equipped with an optical cell (light path:

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1–2 mm). Wavelengths of irradiation of the sample were selected by inserting a glass cut-off filter into the light path between the light source (1 kW Xenon lamp) and the sample. The filters used in the experiment were VO-54, VO-58 and VR-62 (Toshiba) which were confirmed to have no transmission leak at wavelengths shorter than 520, 560 and 600 nm.

3. RESULTS

Fig.1 shows a typical experiment in which the spectrum of hypsorhodopsin was estimated by a modification of the method in [4] in which the difference spectrum of chicken hypsorhodopsin was calculated. As the first step, a difference spectrum between hypsorhodopsin and all-*trans* retinal-oxime plus opsin was calculated by subtracting curve 3 from curve 2. However, the warming and re-cooling gave the sample a frozen state, sometimes different in degree of cracking from the previous state. Therefore, curve 3 is corrected to give the same intensification of absorbance by cooling as that of curve 1; i.e., the ratio of curve 1 to curve 3 in absorbance at 510 nm (an isosbestic point between rhodopsin and isorhodopsin at 9 K) should be equal to that of the corresponding curves at the room temperature in absorbance at 500 nm (the isosbestic point at 298 K). Subtraction of the corrected curve 3 from curve 2, gave a difference spectrum between hypsorhodopsin and all-*trans* retinal-oxime plus opsin. After normalizing the difference spectrum by division with the molar fraction of hypsorhodopsin contained in curve 2, it was added to curve 4, resulting in the absorption spectrum of hypsorhodopsin.

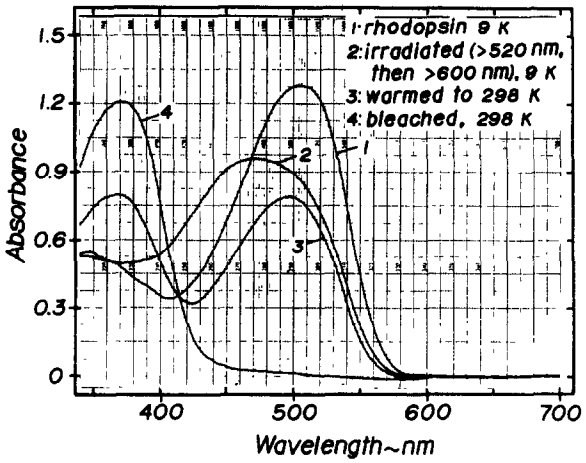

Fig.1. Photoconversion of cattle rhodopsin to hypsorhodopsin at 9 K. Cattle rhodopsin was cooled to 9 K (curve 1), and then irradiated with orange light (>520 nm) for 4 h. The spectrum shifted to shorter wavelengths, indicating a conversion of rhodopsin to a mixture composed of rhodopsin, isorhodopsin and hypsorhodopsin with a small amount of bathorhodopsin. In order to diminish the bathorhodopsin, the preparation was irradiated with red light (>600 nm), until no further spectral change was observed with time, resulting in a mixture of rhodopsin, isorhodopsin and hypsorhodopsin (curve 2). Then the mixture was warmed to 298 K in the dark; this allowed bleaching of the hypsorhodopsin only into a mixture of all-*trans* retinal-oxime (λ_{max} = 367 nm) plus opsin. After cooling to 9 K the spectrum was measured, representing a mixture composed of rhodopsin, isorhodopsin, and all-*trans* retinal-oxime plus opsin (curve 3). Then the mixture was completely bleached with orange light (>520 nm) at 298 K to all-*trans* retinal-oxime plus opsin, followed by measurement of the spectrum at 9 K (curve 4).

Fig.2 shows the absorption spectra of equivalent amounts of rhodopsin and hypsorhodopsin at 9 K,

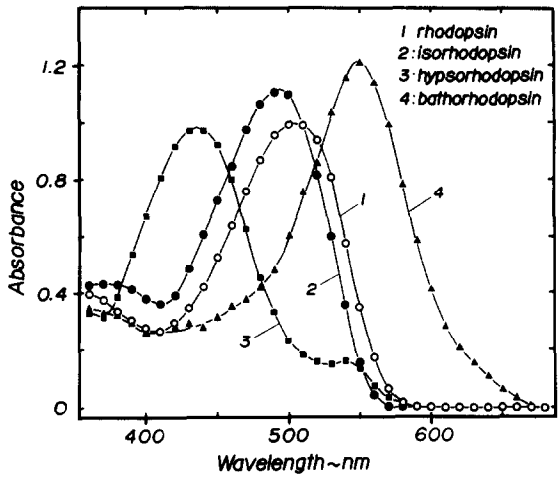

Fig.2. Absorption spectra of cattle rhodopsin (curve 1), isorhodopsin (curve 2), hypsorhodopsin (curve 3) and bathorhodopsin (curve 4) at liquid helium temperatures. The spectrum of rhodopsin (λ_{max} = 506 nm) is brought arbitrarily to maximal absorbance 1.0. Isorhodopsin (λ_{max} = 498 nm), hypsorhodopsin (λ_{max} = 435 nm) and bathorhodopsin (λ_{max} = 548 nm) possess maximal absorbances about 1.12-, 0.98- and 1.22-fold, respectively, as high as that of rhodopsin.

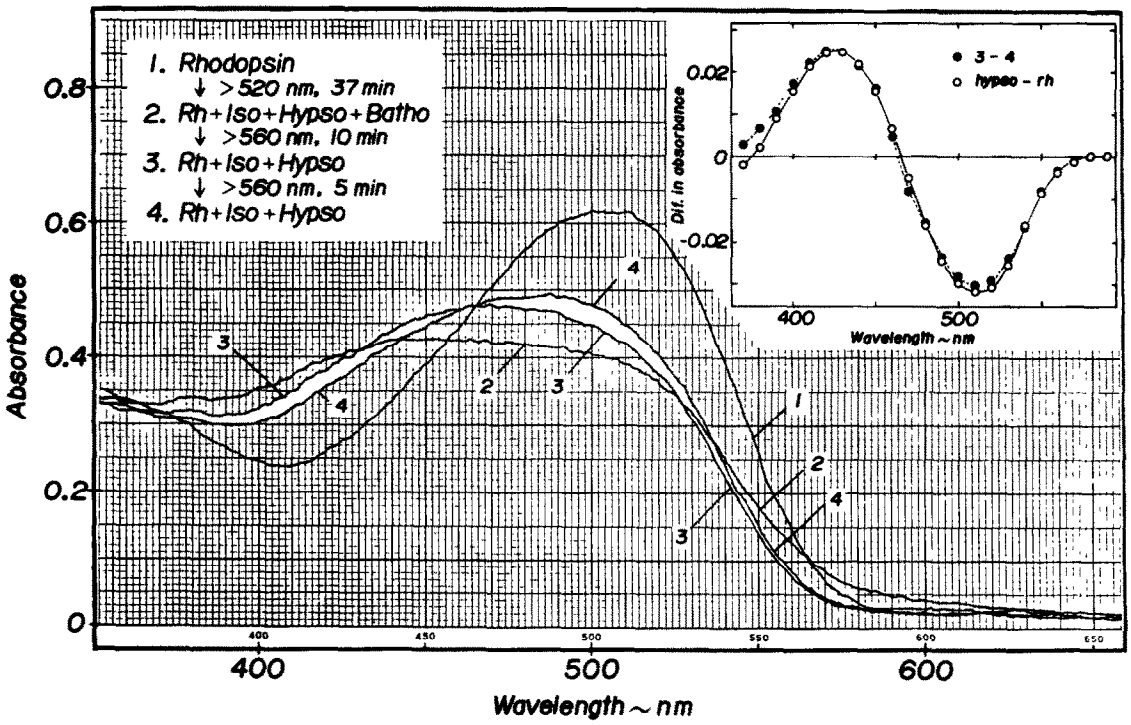

Fig.3. Photoconversion of hypsorhodopsin by irradiation with orange light (>560 nm) at liquid helium temperature (9 K). Curve 1, rhodopsin; curve 2, a mixture of hypsorhodopsin, bathorhodopsin, isorhodopsin and rhodopsin produced by irradiation of rhodopsin with orange light (>520 nm) for 37 min; curve 3, a product of irradiation of curve 2 with another orange light (>560 nm) for 10 min; curve 4, a product of irradiation of curve 3 with the same orange light (>560 nm) for 5 min. Inset: (---●---) difference spectrum between curves 3 and 4; (—○—) difference spectrum between hypsorhodopsin and rhodopsin calculated from curves 1 and 3 in fig.2.

together with those of bathorhodopsin and isorhodopsin. It should be noted that hypsorhodopsin has a main band about 435 nm and a sideband around 540 nm.

In order to further confirm the existence of the sideband, the following experiments were carried out. A mixture composed of rhodopsin, isorhodopsin, hypsorhodopsin and a small amount of bathorhodopsin (fig.3, curve 2) was irradiated with orange light (>560 nm) at 9 K. The irradiation of the mixture resulted in decreases of absorbance in the ranges below 430 nm and above 526 nm and an increase of that between both wavelengths (curves 2 and 3), suggesting that the irradiation mainly caused photoconversion of bathorhodopsin to rhodopsin because the curve intersection point between curves 2 and 3 (526 nm) was very close to the isosbestic point between bathorhodopsin and rhodopsin (523 nm). Further irradiation with the same light caused some photoconversion of hypsorhodopsin to rhodopsin, because the difference spectrum between curves 3 and 4 almost coincided with the difference spectrum between hypsorhodopsin and rhodopsin (fig.3, inset). The small discrepancies between two difference spectra are most likely due to some formation of isorhodopsin. Thus the photoconversion of hypsorhodopsin to rhodopsin with the orange light indicates that hypsorhodopsin may have some absorbance at longer wavelengths than 560 nm.

4. DISCUSSION

Our experiment shows that the absorption spectrum of hypsorhodopsin has a small sideband around 540 nm, which has never been observed before in absorption spectra of other visual pigments and their photobleaching intermediates.

Owing to this sideband, the absorbance of hypsorhodopsin was confirmed to be higher in the range above 550 nm than that of isorhodopsin (fig.2). This fact appears to be a reasonable explanation of the question why the yield of cattle hypsorhodopsin is very low (less than about 40%) [1,2] on irradiation of the rhodopsin at 4–9 K with even long wavelength light, notwithstanding the fact that the λ_{max} of hypsorhodopsin is located at a much shorter wavelength than rhodopsin and isorhodopsin.

It has been suggested that the retinylidene Schiff base of hypsorhodopsin may be deprotonated [2,4,5,7,9]. This suggestion is made on the basis of the fact that the energy difference between hypsorhodopsin and bathorhodopsin calculated from the absorption maxima was almost equal to that between protonated and unprotonated retinylidene Schiff bases (λ_{max} = 438 nm and 365 nm, respectively). This implication was supported by a CNDO calculation on protonated and unprotonated Schiff bases, suggesting that the proton on the retinylidene Schiff base of rhodopsin could come off the imine nitrogen during the photoisomerization from 11-*cis* form to all-*trans* form [10].

If the Schiff base in hypsorhodopsin is not protonated, the lone-paired electron of nitrogen would be excited to the π^* orbital ($\pi^* \longleftarrow n$ transition). However, in general the transition moment of the $\pi^* \longleftarrow n$ transition is so weak in comparison with the $\pi^* \longleftarrow \pi$ transition, that the sideband of hypsorhodopsin would be due not to the $\pi^* \longleftarrow n$ transition, but to the $\pi^* \longleftarrow \pi$ transition.

Recently, theoretical calculations of 2-photon absorptivities for the low-lying $\pi\pi^*$ electronic state have predicted that the lowest excited state of all-*trans* retinal and unprotonated retinylidene Schiff base would be a $^1A_g^-$-like state [11,12]. In polyenes the transition to the $^1A_g^-$-like state is partially allowed by breakage of symmetry, resulting in appearance of the small absorption band at the longer wavelength than the main peak due to the transition to the $^1B_u^+$-like state [13]. Since the retinylidene chromophore in hypsorhodopsin, as well as in bathorhodopsin, has been considered to be distorted [1,2,7], a similar small band would be observed. Therefore, the sideband of hypsorhodopsin should be regarded as a transition to the $^1A_g^-$-like state.

Assuming that hypsorhodopsin has a protonated retinylidene Schiff base just as the chromophore [1,2], the $^1A_g^-$-like state would be expected to be in higher energy level than a $^1B_u^+$-like state, on the analogy of a prediction of the theoretical calculation on the 11-*cis* protonated retinylidene Schiff base [11,12]. A fluorescence experiment of a model Schiff base, *N*-dodecapentaenylidene-butyl amine, however, displayed that the lowest excited singlet of the protonated Schiff base correlated with the forbidden A_g^- states. Accordingly, it might be possible to deduce that the sideband of hypsorhodopsin would be due to a transition to the $^1A_g^-$-like state. The detailed assignment of the two absorption bands of hypsorhodopsin deserves further investigation.

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