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SUGGESTION OF EXISTENCE OF TWO FORMS OF HALORHODOSPIN IN ALKALINE SOLUTION

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SUMMARY Illumination of halorhodopsin (hR_{590}) with orange light in alkaline solution produced a 410 nm absorbing species (hR_{410}), which returned to hR_{590} upon blue light illumination. The amount of the flash-reactive species of hR_{590} was estimated by the flash-yield. Illumination with orange light decreased the flash yield, due to the formation of hR_{410}. Blue light illumination of this sample led to the increase of the yield, which was larger than that before orange light illumination. In dark, the yield decreased gradually in 3 - 4 days. The scheme is proposed in which there exist two forms of hR_{590}.

Cytoplasmic membranes of Halobacterium halobium contain at least three light-reactive proteins whose chromophores are retinal. The first is bacteriorhodopsin(bR) which functions as light-driven H^+pump(1,2). The second is another light-driven electrogenic pump, halorhodopsin (hR)(3,4). Evidence has been presented that hR functions as an inwardly-directed chloride pump on illumination(5,6), although in early studies it was assumed to be a light-driven outwardly directed sodium pump(7). Halorhodopsin absorbs near 590 nm and undergoes a photocycle with a photoproduct absorbing at 490 nm whose half time of decay is about 10 msec(8,9). The third light-reactive protein is called third rhodopsin-like pigment (tR) or slowly cycling rhodopsin (sR), whose photocycle is completed in a few seconds, while that of bR and hR is completed in tens of msec(8,10). Bacteriorhodopsin was the first to be discovered, and it has been studied well in the last ten years. In contrast, characterization of hR and tR has begun quite recently.

Previous work(11-13) has shown that there exists a 410 or 420 nm absorbing species as one of photoproducts of hR, although it was not detected by flash photolysis(8). This species will be referred to as

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hR_{410}, hereafter, and the original pigment of hR absorbing ca 590 nm is as hR_{590}. In the present communication, we present the data showing that in alkaline solution illumination with orange light, which is absorbed by hR_{590}, decreases the flash(>630 nm) yield due to the formation of hR_{410} and that blue light (peak wavelength 390 nm) illumination recovers it. The flash yield represents the relative amount of the flash-light reactive species of hR_{590}. Interestingly, the flash yield of the sample after the blue light illumination was found to be larger than that before formation of hR_{410}. After incubation in the dark, the flash yield decreased. We propose the scheme that hR_{590} is composed of two forms in dark, one is reactive to the flash light while the other is not.

MATERIALS AND METHODS

The strain used was KH-10 (bR^-,hR^+,tR^+,carotenoid$^-$ see ref.8). The procedure of the growth of bacteria and the preparation of envelope vesicles were described earlier(8). The pH of the solution was adjusted with 50 mM of following buffers: 2-(N-morpholino) ethanesulfonic acid below 7, tris(hydroxymethyl)aminomethane for pH ranging from 7 to 9.5, and 3-cyclohexylaminopropanesulfonic acid above 9.5. Protein was assayed by Lowry method using bovine serum albumin as a reference standard.

The set-up of the flash photolysis and the procedure were the essentially the same as used previously(flash lamp >630 nm, duration of 200 μsec. ref.8). The wavelength of measuring beam was 590 nm. The experiments were carried out with 4M NaCl at 20 ^{o}C. The data obtained were analysed by the following equation:

$$\Delta A_{590}(t) = \Delta A^{hR}\exp(-k^{hR}t) + \Delta A^{tR}\exp(-k^{tR}t)$$

where k^{hR} and k^{tR} were about 70 and 0.89 sec^{-1}, respectively. The value of ΔA^{hR} and ΔA^{tR} is referred to as flash-yield. The difference spectrum was measured with Shimazu UV-300 (Shimazu, Kyoto) equipped with an end-on photomultipler. Orange light was obtained with use of an orange filter (>530 nm, Toshiba VR53) and a projector lamp. To obtain blue light, a band-pass filter (peak wavelength 390 nm, Toshiba C39) was used.

RESULTS AND DISCUSSION

In alkaline solution (pH 7 -10), orange light illumination (330 W/m^2) led to a gradual decrease in the flash yield of hR, ΔA^{hR}, for 15 - 30 min. The decrease was pronounced as the pH in the medium and the light intensity increased. This decrease is due to the formation of hR_{410} (see below). The necessity of long illumination with high intensity suggests that the quantum efficiency of the formation of hR_{410} is so small that we cannot observe its formation by flash photolysis. The values of ΔA^{tR}

were not changed during illumination. The pigment of tR can be bleached with hydroxylamine(13). When the bleached sample was used, the same results described below were obtained, showing that the results obtained are due only to hR. The difference spectrum between once orange light illuminated and un-illuminated samples (which had been incubated in dark for 3 days) shows the formation of hR_{410} with concomitant decrease in the absorbance at 590 - 580 nm (depletion of original pigment), as in ref.13 (see solid line of Fig,1).

The recovery of the flash yield was very slow in the dark (half-time was several hrs). But, blue light illumination accelerated the recovery very much. Illumination of hR_{410} with blue light for several minutes increased the flash yield, as shown in Fig. 2. Moreover, upon illumination of blue-light, the disappearance of hR_{410} and the formation of a species absorbing at 590 - 580 nm were observed (see broken line in Fig.1). These facts indicate that blue light illumination regenerates the original pigment from hR_{410}. The half time of the regeneration was

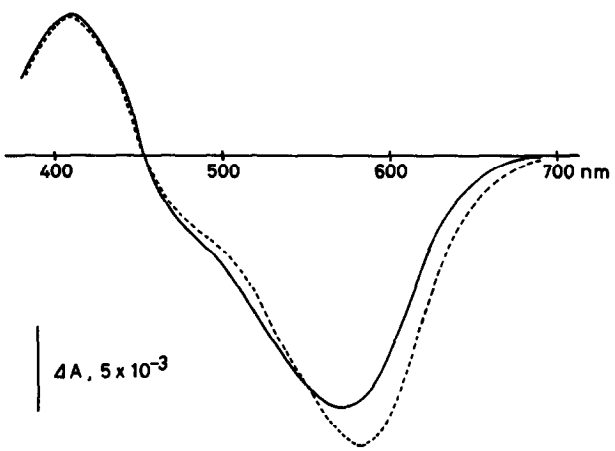

Fig.1. Formation of hR_{410} by orange light illumination and regeneration of hR_{590} by illumination of hR_{410} with blue light. Solid line: difference spectrum between the sample incubated in dark for 3 days at 20 °C and orange-light illuminated one. Broken line: difference spectrum between the orange-light illuminated and orange and subsequent blue light illuminated samples. Note that the polarities of solid and broken lines are opposite each other in order to make clear the shift of the maximum wavelength at 590 - 570 nm. For both cases, orange light was illuminated for 20 min with intensity of 330 W/m^2. The blue light (50 W/m^2) was irradiated for 10 min. Light intensity was measured with YSI-Kettering radiometer (Model 65A). KH-10 vesicles of 2.5 mg protein/ml were suspended in 4 M NaCl of pH 9.0.

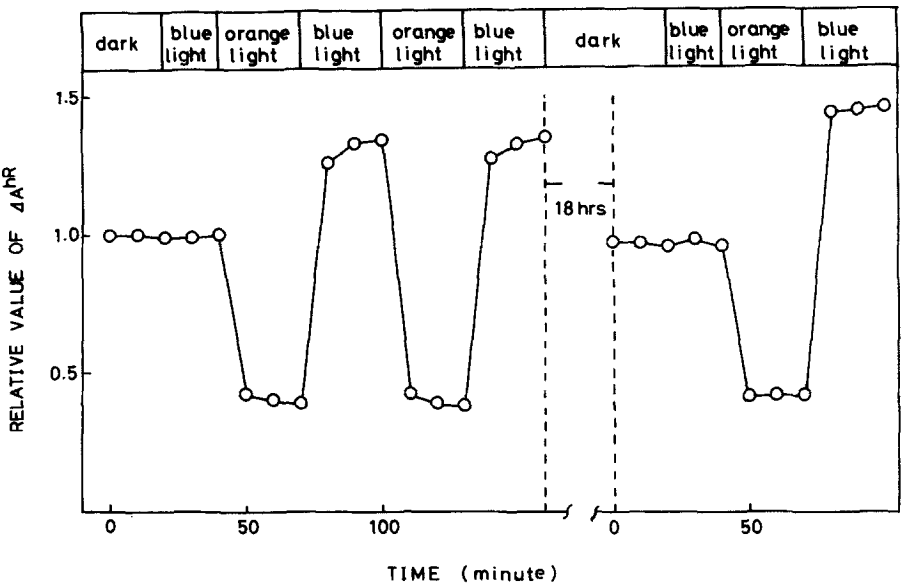

Fig.2. Overshoot of the flash-yield by the illumination of orange and subsequent blue light. Sample (2.5 mg protein/ml) was suspended in 4 M $NaCl_2$ of pH 9.0. Light intensities of orange light and blue light were 330 W/m^2 and 50 W/m^2. The incubation in the dark between the experiments was carried out at 20 °C for 18 hrs.

determined to be about 0.4 sec by flash photolysis (data not shown). It should be noted that the flash-yield of the sample after orange and subsequent blue light illumination exceeded that of the sample before orange-light illumination. Illumination of the sample in the "higher flash-yield" state with orange light led to the decrease in the flash yield, whose value was approximately the same as before. Illumination of this sample with blue light brought the state of higher flash-yield, and this cycle was possible to repeat many times. When the sample of higher flash-yield state was incubated in the dark, the flash yield gradually decreased and became a steady level after about 3 days (whose relative flash yield was about 0.7). This decrease did not correspond to denaturation of the pigment, because orange and subsequent blue light illumination regenerated the state of high flash-yield. Therefore, we assume that the state of higher flash-yield changes to another form in the dark, which shows no or less response to the flash. This form is not hR_{410}, because blue light illumination exerted no effect on the increase of the flash yield of the sample which had been incubated in the dark.

From these observations, it is inferred that hR_{590} is composed of two species, ie hR_α and hR_β (see Fig. 3). The form of hR_α is sensitive to flash light and exhibits a photocycle with the photo-transient absorbing at 490 nm. On the other hand, the species of hR_β is not or less sensitive to the flash. Illumination with orange light in alkaline solution transforms hR_α into hR_{410}, which gives rise to the decrease in the flash-yield. The reversal of the change was very slow in the dark, but blue light accelerated it. Thus, blue light produces hR_α from hR_{410}. hR_α transforms gradually into hR_β in the dark, which leads to the decrease in the flash-yield. Since the rate of transfomation from hR_α to hR_β is assumed to be slow in the dark(it took about 70 - 90 hrs), the rate constant of the reverse reaction in the dark is considered to be small; Othewise, hR is present mainly as hR_α.

The overshoot of the flash-yield after illumination with blue light can be explained as follows: The concentration of hR_α becomes larger than that before orange-blue light illumination. In other words, hR_β is transformed into hR_α during the process of orange-blue light illumination. One possibility is that blue-light illumination transforms hR_β to hR_α. But this is not the case because Fig. 2 shows that blue light alone has no effect on the sample incubated in the dark. Note that blue light increases the flash yield only for the sample illuminated by orange light.

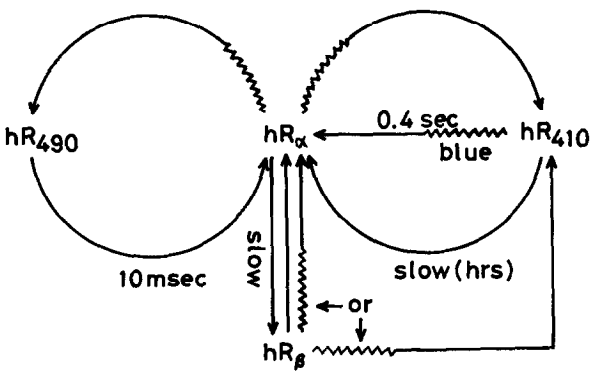

Fig.3. Tentative scheme of halorhodopsin in alkaline solution. The arrows (⟿) without any notation mean the pathway which is due to orange light. See text for details.

Therefore, we have to assume a pathway from hR_{β} to hR_{α} or to hR_{410}, which requires orange light. At present, the question which path is correct cannot be answered. If there exists light-dependent pathway from hR_{β} to hR_{α}, it suggests that hR also has light-adapted (hR_{α}) and dark-adapted (hR_{β}) forms as bR does(2).

According to this scheme, the broken line in Fig.1 is the difference spectrum between hR_{410} and hR_{α}, indicating that the max. wavelength of the absorption of hR_{α} is about 583 nm at pH = 9. The solid line in the figure is the difference spectrum between hR_{410} and the mixture of hR_{α} and hR_{β}. The peak is blue-shifted by 10 nm from that of the broken line, indicating that the max. wavelength of the absorption of hR_{β} is blue-shifted from that of hR_{α}. Further study will be needed to determine these wavelength precisely.

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