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LIGHT-DEPENDENT PROTON MOVEMENT AND PHOTOINTERMEDIATES ON BACTERIORHODOPSIN PHOTOCYCLE

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Abstract: The relationship between photointermeidates of bacteriorhodopsin (bR) and proton uptake was investigated by a flash photolysis technique. Attention was paid to keep the sample in a light-adapted state and under the same conditions during measurements. The decay of M-intermediate (M), the formation and decay of O-intermediates (O), the recovery of bR and the release and the uptake of protons were measured with a purple membrane suspension (pH 7.0, 0.5 M NaCl) at various temperatures.

The following reaction scheme for the bR photocycle was deduced from the results. 1) The decay process of M consists of three components (M_{f1}, M_{f2} and M_s). 2) O is formed from the faster component of M (M_{f1}). 3) A part of M (M_{f2} and M_s) decays directly to bR without passing through O. 4) The uptake of protons is coupled with the decay of the slowest M (M_s).

The above reaction scheme was confirmed by the results obtained from similar experiments using an analog pigment of bR, Np-bR, M of which decays much more slowly than that in native bR system.

Key words: Purple membrane, Proton pump, Photocycle, Intermediate, Reacition pathway, Temperature dependence,

1. INTRODUCTION

In attempts to elucidate the molecular mechanism of light-dependent proton pumping, bacteriorhodopsin (bR) has been a subject of considerable interest (1-4). The mechanism, however, is not fully understood yet, because several discrepancies remain. One of them is a decay pathway from M-intermediate (M). Many different studies have been performed on this problem. Many authors found a biphasic decay of M under several different conditions (5-9). Recently, Groma and Dencshazy (10) observed three components in the M decay of cell envelope vesicles in 4 M NaCl, and some authors reported a new molecular species related to M (11, 12). The other

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remaining discrepancy is a relation between an intermediate and proton transport. Some have reported that there was not a good kinetical correlation between the photointermediate and proton movement (5) and some reported that proton re-uptake correlated with the slow decay of M (6, 13, 14).

In present study, we measured the decay process from M at several wavelengths and temperatures under the constant pH and salt conditions (pH 7.0, 0.5 M NaCl). We also measured proton movement within the same time range. Attentions were paid to keep the sample in a light-adapted state and measure automatically under the same conditions. A model was deduced from the results, for the decay pathway from M and the relation between proton uptake and photointermediate, and tested using an analog pigment of bR synthesized from naphthylretinal and bacterioopsin, Np-bR (15), M of which decays more slowly than that of bR. In both systems the decay of M consisted of three components and the slowest M coupled to the proton uptake.

2. MATERIALS AND METHODS

Halobacterium halobium was cultured and purple membrane (PM) was prepared according to Becher and Cassim (16). The purple membrane was suspended in 0.5 M NaCl solution and the pH was adjusted to 7.0 ± 0.1 just before measurements.

The preparation of bleached purple membrane (bPM) was according to Tokunaga and Ebrey (17). The bleached membrane was suspended in 10 mM HEPES buffer (pH 8.0). On the preparation of Np-bR, about one tenth molar amount of the purified naphthylretinal (all-(E)-3-methyl-7-(2-naphthyl)-2,4,6,-octatrienal; kindly presented by Dr. K. Tsujimoto) was added successively to the bPM suspension at two-hour intervals (15). After the pigment was fully regenerated, it was incubated in the dark at 4°C for several days to change the pigments completely into a single species, Np-bR having λ_{max} at 503 nm.

Absorbance changes due to photointermediates or proton movement were measured with the home-made automatic laser flash photolysis system. The system is controlled by micro-computer (Apple II). The sample in the temperature-controlled cell was irradiated with light (530 nm) from a W-lamp to make the sample light-adapted. Five hundred ms after cutting off the adapting light, the sample was excited with the laser pulse (Phase R, 500 nm, pulse width; ca. 600 ns). The absorbance change was measured for an appropriate period and stored in the transient memory. Then the sample was again irradiated with the adapting light and the signal was transferred to the micro-computer. This cycle was repeated for desired times and the signals were accumulated. Thus, the sample was always kept in a light-

adapted state.

Formation and decay of the photocycle intermediates were measured by absorbance change at the following wavelengths; the decay of M was monitored at 400 nm, recovery of bR at 570 nm, and the formation and decay of O at 660 nm. In order to analyze the decay of M or the recovery of bR, the time course of the absorbance change was fitted to the following equation.

$$A(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(-k_3 t)$$

The third term of the right hand of the equation is usually zero except for the measurements with Np-bR. The time constant for the decay of O is not so different from that for the formation. Therefore, the absorbance change at 660 nm was fitted to the following equation.

$$A(t) = A_4 \exp(-k_4 t) - A_5 \exp(-k_5 t)$$

The k_4 and k_5 represent the rate constant for decay and formation of O, respectively. A_5 represents the absorption difference at 660 nm between O and its precursor. A_4 represents the absorption difference at 660 nm between O and the decay product of O, probably bR.

The proton uptake from the medium was monitored by the absorbance change at 610 nm with or without pH-indicator dye, Bromothymol blue; BTB. BTB changes its λ_{max} from 430 nm (pH 6.0) to 610 nm (pH 8.5) depending on pH. In PM suspension (0.1 M HEPES, 0.5 M NaCl, pH 7.0), BTB did not change the time course of M decay and the molar ratios of M species. This indicates that BTB does not affect photochemical properties of bR. The pH-dependent color change of BTB was not affected by presence of PM. When BTB was washed out from the medium, the signal derived form BTB disappeared easily, indicating that BTB is not strongly adsorbed by PM. Thus, It was concluded that BTB should not interact with PM and not affect the photochemical property of P.M. The absorbance change observed by irradiation should be resulted from proton movements to and from the water phase. On measuring the proton movements, 127μl of 0.04 % BTB solution was added to one ml of PM suspension (A_{570} = 1.0 in 0.5 M NaCl solution), and the pH of the suspension was adjusted to 7.0 ± 0.1. The molar ratio of BTB to bR is about 5, where the absorbance change of the dye is not dependent on its concentration. After the measurements without BTB, BTB solution was added to the sample. Then the absorbance change at 610 nm was again measured. Taking account of the intensity of the exciting flash and the concentration of the sample, the absorbance changes due to proton release and uptake were estimated. The time course for proton uptake was fitted to the following equation.

$$A(t) = A_6 \exp(-k_6 t)$$

3. RESULTS AND DISCUSSION

Measurements using native PM were performed at several temperatures and are summarized in Figs. 1 and 2. The decay process of M was fitted with two exponentials, M_f and M_s. The decay of M_f occurred in the same time range as the formation of O at all temperatures, strongly suggesting that M_f changes to O. The decay of M_s was in the same time range as the decay of O, not as the formation of O. From this result, it can be concluded that M_s cannot change to O. The uptake of protons occurred later than the decays of M_f, M_s and O. The changes in absorbance reflect those around chromophoric retinal occurred inside the protein, but the uptake of protons occurs on the membrane surface. So it is not necessary that the decay time course of intermediate should be equal to that of the uptake of protons. A good correlation between both process is not observed in Fig. 1.

Figure 2 shows the change of the amount of each component at several

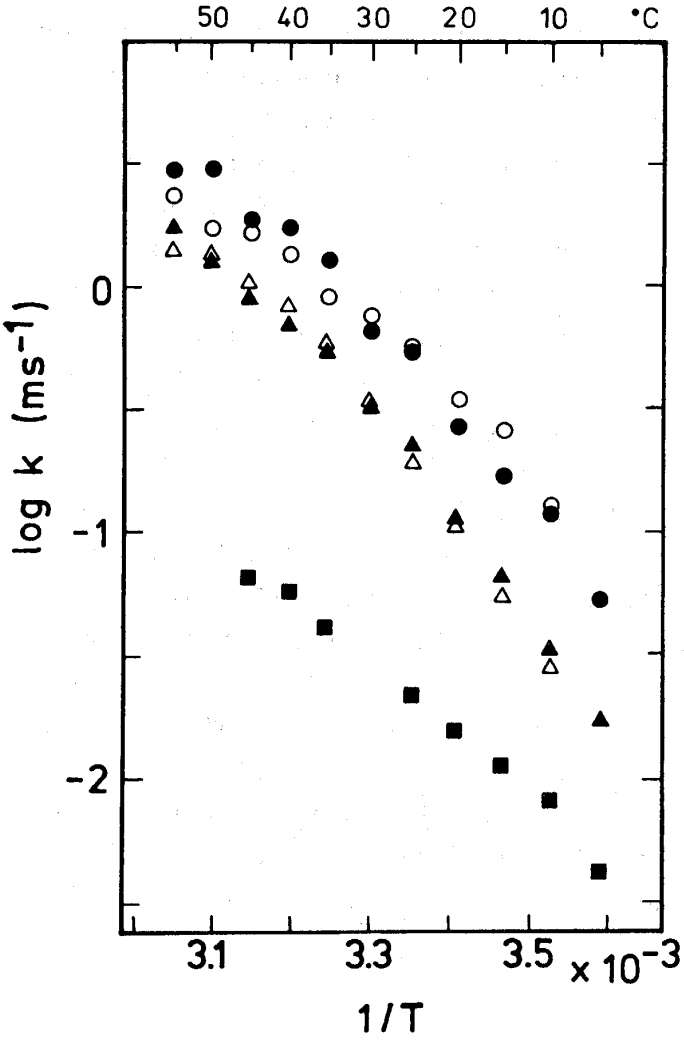

Fig. 1. Arrhenius plot of the rate constants of intermediates of bR and proton uptake.
●, M decay fast; ▲, M decay slow; ○, O formation; △, O decay: ■, proton uptake.

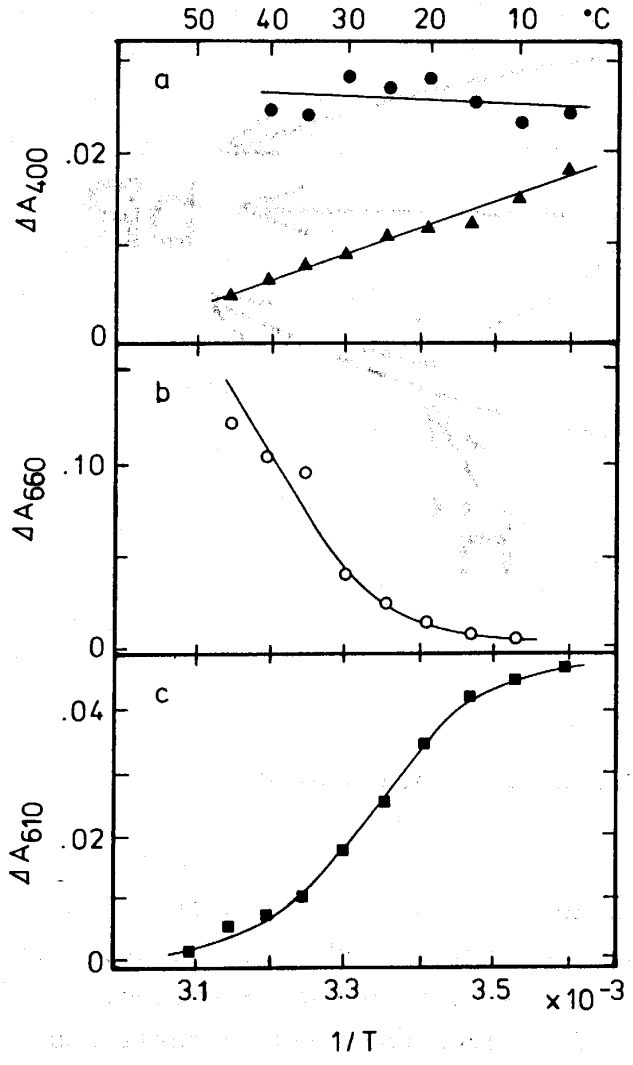

Fig. 2. Temperature dependence of the amount of each component.
a; ●, M decay fast;
▲, M decay slow.
b; ○, O
c; ■, proton uptake.

temperatures. The amount of M_f increased slightly with a rise of the temperature from 5 to 40° C, although that of M_s decreased to less than a half the amount of that observed at 5° C. The absorbance change at 660 nm corresponding to that of O was scarcely observed at temperatures below 10° C, and increased with rises of the temperature. The amounts of both M_f and O increased with rises of temperature. The amounts of both M_f and O increased with rises in temperature. The change in the amount, however, is much larger in O than that in M_f. The kinetic measurements (Fig. 1) suggest that M_f changes to O. The temperature dependence of the yield, however, did not coincide well with that of the rates. If all M_f change to O, the extinction coefficient of O should depend on temperature, and increase to much larger values at higher temperatures. It seems, however, unlikely that the extinction coefficient increases so tremendously. Another explanation for the result is as follows: M_f is composed of two species, M_{f1} and M_{f2}. When the temperature goes up, the amount of M_{f1}

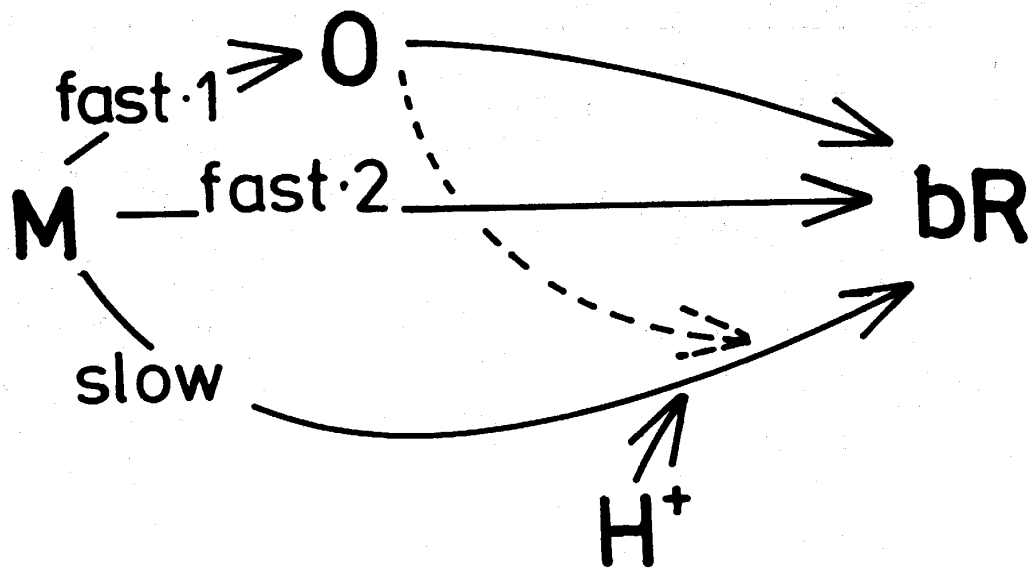

Fig. 3. The proposed model for decay process of M. Details are given in the text.

decreases and that of M_{f2}, which changes to O, increases (Fig. 3). The following experiments with the analog pigment of bR, Np-bR, strongly suggest that the latter explanation is the case in the bR system.

The absorbance change at 610 nm decreased when temperatures rose to 35 from 15° C. This indicates that the amount of protons released and then re-uptaken decreased at higher temperatures. The amount of M_s showed similar dependence on temperature, but the amount of M_f and O did not (Fig. 2). The amount of M_f looks like temperature-independent and that of O increased at higher temperatures.

The measurements using Np-bR were done at 10° C, because at higher temperatures Np-bR having λ_{max} at 503 nm changes to another molecular species, Np-bR_{442}, which has λ_{max} at 442 nm and lesser activity for proton pumping (our unpublished result). The results are summarized in Table 1. The decay of M of Np-bR (Np-M) composed of three species. Two faster-decay Ms had life times of about $1x10^{-2}$ and $5x10^{-2}$ s, respectively. The most stable M had a life time of second range (8 s). We thought they correspond to M_{f1}, M_{f2} and M_s in the native bR system. The proton uptake was observed accompanying a decay of Np-M_s in second range. The amount of protons moved was about an half in Np-bR system as that in bR system. This result is

consistent to the previous one that obtained from the reconstituted-vesicle system (15). The other two M's, which decay in millisecond range, Np-M_{f1} and Np-M_{f2}, did not show uptake of protons.

Np-O was formed after the decay of Np-M_{f1} with a half time of 2×10^{-2} s and decayed after that of Np-M_{f2} with a half time of 5.5×10^{-1} s. The recovery of Np-bR was not observed with the decay of Np-O, but was observed with the decay of all Np-M-species. In the case of the bR system the recovery of bR was observed accompanying the decay of both M-species and the decay time of O was similar to that of M_s. Thus, so far, it has not been possible to elucidate what the decay product of O is. Two candidates are 1) bR or Np-bR, and 2) M_s.

Some reports that M_f and O are in equilibrium. If M_f and O are in equilibrium, the decay half time of O should be same as that of M_f. But that is not the case in our measurements. The formation of O does not

Table 1. The half times (ms) for the decay of M and proton uptake of the bR and Np-bR systems at 10° C.

	M		bR or Np-bR	O		H^+
	formation	decay	recovery	formation	decay	uptake
bR	0.3	8.0 (0.027) 28 (0.068)	7.3 (0.018) 29 (0.133)	5.8	33 (0.008)	78 (0.029)
Np-bR	0.15+0.02	13+2 (0.023) 51+5 (0.021) 9.1+3 x 10^3 (0.016)	11+1.5 (0.023) 49+4 (0.024) 9.9+3 x 10^3 (0.023)	22+1	5.5+3 x10^2 (0.005)	11+3 x10^3 (0.008)

"Recovery" means the recovery of the mother pigment. The number in parenthesis is the yield of each component in absorbance unit. In the bR system each component was monitored at the wavelength described in Materials and Methods. In Np-bR system, the formation and decay of M were monitored at 380 nm. Those of O were at 590 nm and the recovery of Np-bR was monitored at 510 nm. The uptake of protons was monitored at the same wavelength as in bR system. Three sets of recordings were normalized and given in the table. One recording is composed of 8 shoots to avoid formation of another molecular species by light.

occur at the same time as the formation of M (Fig. 1 and Table 1). The conversion from M_f to O should be a very slow process, if M_f and O are in equilibrium.

It is more likely, therefore, that M_f changes to O and that O does not change to M_f but to M_s or bR. In the Np-bR system, the ratio of recovered Np-bR to decayed Np-M is a little smaller in Np-M_{f1} than those in Np-M_{f2} and Np-M_s. This would suggest that a part of Np-M_{f1} changes to Np-O.

The above results obtained from the Np-bR system support the photocycle shown in Fig. 3. M-species are composed of three different species, M_{f1}, M_{f2} and M_s. M_{f1}, the decay of which is the most rapid, changes to O. In the case of the Np-bR photocycle, a part of this component changed directly to Np-bR without formation of Np-O. It is not clear at present whether or not this phenomenon is observed only in the Np-bR photocycle. There are the other M-species, M_{f2} and M_s, which do not form O species and revert to bR directly. The decay rate of M_{f1} and M_{f2} should be similar in the bR system. The proton uptake was observed only accompanying the decay of M_s, not with M_{f1} and M_{f2} in both systems.

Grzesiek & Dencher (14) investigated the stoichiometry and transport kinetics of the light-driven proton pump and reported that proton re-uptake parallels the slow decay process of M or the decay of O-640. Li *et al.* (13) suggested O-640 and M_s as candidates for coupling of proton uptake and photocycle, and concluded that M_s is more likely because of the similarities in the pH dependency of relative quantum yield and temperature dependency of the yield. The present work with PM suspension indicates that proton uptake couples the slow decay of M, which is consistent with Li *et al.* (13). The above conclusion was confirmed using Np-bR.

Another conclusion of the present work is that the decay process of M consists up of three components. The model shown in Fig. 3 is similar to that proposed by Renald and Delmelle (18). Recently, two more reports have supported the conclusion (10, 11).

In our experiments using Np-bR, Np-O did not decay in the same time range as Np-M_s (see Table), though decay times of both M_s and O are quite similar in PM suspension (see Fig. 1). From this result it seems not to be likely that O and M_s are in simple equilibrium. We did not observe any increase in absorbances at 560 nm accompanying the decay of O (Table 1). This is one of the remaining problems in the photocycle of bR. The other is to clarify the differences among "M" species on a molecular basis. One possible origin of three M-species is the difference in the structure of chromophoric retinal; syn-anti at C15=N (19) and cis-trans at some single bond (20). According to Smith et al. (19), 15-trans structure is important to pump protons. Thus, the difference in the bond structure of C15=N may result in the difference in the decay rate. And also the difference in the

bond structure of 14-s would result in the presence of O. The M-species which has 14-s-cis structure should isomerize two bonds on the way back to the original bR, 13 and 14-s. The isomerization of 14-s-cis bond may result in formation of O-intermediates. It should be urgently needed to clarify the chromophoric structure of M and O.

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