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Association between a M-lacking mutant D75N of *pharaonis* phoborhodopsin and its transducer is stronger than the complex of the wild-type pigment : Implication of the signal transduction

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In halobacterial membrane, *pharaonis* phoborhodopsin (or *pharaonis* sensory rhdopsin II, *ps*RII) forms a complex with its transducer *p*HtrII. Flash-photolyis of D75N mutant did not yield M-intermediate but an O-like intermediate is observed. We examined the interaction between D75N of *p*pR and t-Htr (truncated *p*HtrII). These formed a complex in the presence of n-dodecyl-β-D-maltoside, and the association accelerated the decay of the O of D75N from 15 to 56 s^{-1}. From the decay time constants under varying ratios of D75N and t-Htr, n, the molar ratio of D75N/t-Htr in the complex, and K_D, the dissociation constant, were estimated. The value of n was unity and K_D was estimated to 146 nM. This K_D value can be considered as the association between the photo-intermediate and t-Htr, which is deduced by the method of estimation. Previously we (*Photochem. Photobiol.* 74, 489-494 (2001)) reported K_D of 15 μM for the interaction between the wild-type and t-Htr by means of the change of M-decay rates. Therefore, this value should be the K_D value for the interaction between M of the wild-type and t-Htr.

Key words : Sensory rhodopsin, protein-protein interaction, photocycle, flash-photolysis, M-intermediate

INTRODUCTION

pharaonis phoborhodopsin (*p*pR) absorbs maximally 498 nm light, and work as receptor of the negative phototaxis [1]. *p*pR transmits signals through an integral membrane halobacterial transducer protein (*p*HtrII). *p*HtrII has both a signaling domain and two methylatable domains such as an aspartate receptor (Tar) in *Escherichia coli*, and activate a phosphotransfer cascade producing flagella motor responses. By these signaling systems, bacterial cells avoid a shorter wavelength light (λ<520 nm) that contains harmful near-UV light.

We [2] succeeded in an expression of a truncated *p*HtrII (named as t-Htr) in *E. coli*, where t-Htr is a N-terminal sequence of 159 amino acid residues of *p*HtrII. This t-Htr has the ability of contact with *p*pR under solubilized conditions with n-dodecyl-β-D-maltoside (DM) [2,3]. t-Htr are proposed to interact physically and functionally with *p*pR via helix-helix contacts between their transmembrane segments [4]. Thus, *p*pR/t-Htr complex is considered to serve as an adequate model system to elucidate the signal transfer.

Our previous paper [2] showed that M-decay of the complex between the wild-type *p*pR and t-Htr becomes about twice slower than that of *p*pR alone. From the M-decay rate data during the titration of t-Htr with the wild-type *p*pR, the dissociation constant, K_D of the complex was estimated to 15 μM. On the other hand, Wegener suggested K_D of 100 nM in the dark [5]. What is a reason for this great difference between 15 μM and 100 nM? One possible explanation is that the complex forms firmly in the dark while at the M-state the affinity of the complex becomes weak, because this value was estimated from the M-decay data of the complex between the wild-type *p*pR and t-Htr. This assumption undertakes the present work where the interaction between D75N mutant and t-Htr is investigated using flash-photolysis, because D75N mutant lacks for the M-intermediate and has O (or N)-like intermediate as an only main intermediate in ms time range [6].

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MATERIALS AND METHODS

Sample preparations. The expression of the histidine-tagged proteins in *E. coli* BL21 (DE3) and purification of proteins were described elsewhere [2,7]. Sample medium was exchanged by ultafiltration (UK-50, Advantech, Tokyo) and samples were finally suspended in a buffer solution containing 400 mM NaCl, 10 mM Tris-HCl (pH 7.0) and 0.1% DM (n-dodecyl-β-D-maltoside).

Flash spectroscopy. Apparatus and procedure were essentially the same as described previously [2]. A photo-intermediate was observed at 570 nm and its time-course was analyzed by a single exponential equation to determine the kinetic constant.

Titration of free t-Htr with D75N and estimation of binding parameters. Under the condition that t-Htr concentration was kept constant at 25 μM, varying concentrations of D75N were added to change the molar ratio of t-Htr to D75N and we measured the kinetic constant of the intermediate by flash spectroscopy as described above. We estimated the binding parameters using the same method described elsewhere [2].

RESULTS

D75N lacks the M-intermediate during the photocycle because Asp-75, the proton acceptor from the protonated Schiff base, is replaced by the neutral Asn [6]. In ms time range, a red-shifted O (or N)-like intermediate (λ_{max} of 570 nm) is observed. Adopting a schema [6], the photocycle of D75N may be described as: D75N*pp*R_{520} $\rightarrow$ K_{565} $\rightarrow$ Z_{570} $\rightarrow$ D75N*pp*R_{520}. The suffix indicates λ_{max}. They considered Z_{570} as a mixture of two intermediates. The characterization of Z_{570} await for a further investigation, but it should be stressed that M does not appear and that O-like intermediate is only observable except early time range after the flash. As shown in insets in Fig. 1, the logarithmic plot suggests the presence of an earlier intermediate with the life-time of several ms, which might be K_{565} because Schmies *et al.* [6] reported the half-time of 7 ms.

Curve (a) in Fig. 1 is the decay trace of free D75N and curve (b), that of D75N/t-Htr complex. Insets delineate log of absorbance changes against the time after the flash. These traces were analyzed by a single exponential equation to estimate the time constant of Z_{570}, because the contribution from the earlier component is very small. In addition, Z_{570} is a slow component and

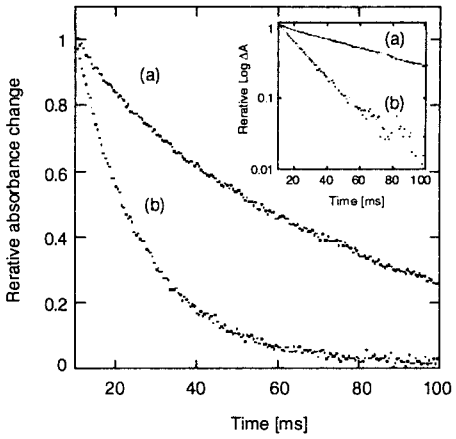

Figure 1. The decay rate of a photo-intermediate is different between free D75N (a) and D75N/t-Htr complex (b). Protein concentrations of D75N were 5 μM for both (a) and (b). Insets show the logarithmic plot of (a) and (b).

semi-logarithmic analysis may give an exact time constant for the slower component. The rate constant of D75N alone was 15 ± 0.1 s^{-1} while that of the complex, 56 ± 0.3 s^{-1}. The decay of Z_{570} of the complex is almost 4-fold faster than D75N alone. No change in the decay rate of Z_{570} was observed when additional t-Htr was added to the D75N/t-Htr complex, implying that there was no free D75N in the sample.

We titrated t-Htr of 25 μM with D75N and measured the decay rate of Z_{570}. Eight kinetic traces were obtained under the condition of varying molar ratio of t-Htr to D75N. All data were fitted well with an equation of $\alpha\exp(-15t) + \beta\exp(-56t)$.

We must consider extinction coefficients of Z_{570} derived from both D75N and D75N/t-Htr, which were assumed to be equal each other. Under this assumption, α and β in the above equation are proportional to the concentration of D75N alone and D75N/t-Htr complex, respectively. This assumption was deduced from the followings. 1) The extinction coefficients of the ground state of D75N and D75N/t-Htr are the same (data not shown). 2) In the flash-induced light-dark difference spectrum, the deflection ratios of the positive band caused by the formation of Z_{570} to the negative band caused by the depletion of the original pigments are equal (data not shown).

In Fig. 2, the free D75N concentration, [D75N], is plotted against the D75N/t-Htr concentration, [D75N/t-Htr]. From this curve, K_D and n values were estimated to 146 nM (± 8) for K_D and 1 (± 0.001) for n.

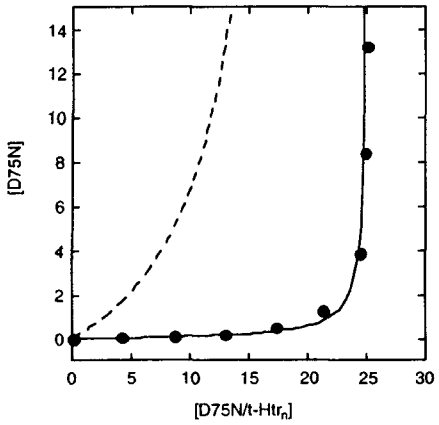

Figure 2. The concentration of the free D75N, [D75N], is plotted against the concentration of D75/t-Htr$_n$ complex, [D75N/t-Htr$_n$], during the titration. These concentrations are expressed in an unit of μM. Closed circles are data points and solid line is a fitting curve calculated by a method described previously [2]. Broken line indicates the interaction between M of the wild-type *p*pR and t-Htr [2].

DISCUSSION

This paper shows followings; 1) The value of n is unity, indicating the (1:1) stoichiometry of D75N and t-Htr as is the same with the wild-type [2]. 2) The K_D value is 146 nM. Previously, we [2] showed K_D of 15 μM, which was estimated from the decay rate constants of M-intermediate of *p*pR and *p*pR/t-Htr complex. This value, hence, should be considered as K_D at the M-state of the complex. On the other hand, Wegener presented an interesting result on the interaction of the wild-type *p*pR and t-Htr. Using an iterative titration calorimetry, they observed K_D of about 100 nM in the dark [5]. It is of interest that this value is very close to 146 nM for the intermediate of D75N that lacks M in the photocycle, and is almost 100-fold smaller than that for the interaction between M of the wild-type and t-Htr.

Wegener *et al.* [8] showed a photo-induced rotation of TM2 of t-Htr, which results in the decrease in the association affinity (decoupling). This is consistent with the low affinity of the M-intermediate as was reported previously [2]. The photo-induced changes in the EPR signal continued till the full recovery of the original pigment [8], which seems to be contradictory to the present data in which the association affinity of the O-intermediate (146 nM) is reset to the stronger value of the ground-state *p*pR provided that Z_{570} corresponds to the O-intermediate of the wild-type. Further studies are necessary on the association affinity of each M or O (or N)-intermediates with the transducer, and on the question of what is the signaling state in *p*pR.

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