Association of *pharaonis* Phoborhodopsin with its Cognate Transducer Decreases the Photo-Dependent Reactivity by Water-Soluble Reagents of Azide and Hydroxylamine

By

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Key words: Bleach of retinoid protein, flash photolysis, sensory rhodopsin, *Halobacterium salinarum, Natronobacterium pharaonis*

Running title: Structural Changes induced by the association with transducer

Abbreviations: DM, n-dodecyl-β-D-maltoside; *p*HtrII, *pharaonis* halobacterial transducer of *p*pR; *p*pR; *pharaonis* phoborhodopsin (*pharaonis* sensory rhodopsin II); t-Htr, truncated *p*HtrII; MRT, mean residence time; ppR$_M$, M-intermediate of *p*pR; ppR$_O$, O-intermediate of *p*pR

ABSTRACT

pharaonis phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, *p*sRII) is a receptor of the negative phototaxis of *Natronobacterium pharaonis*. In bacterial membrane, *p*pR forms a complex with its transducer *p*HtrII, and this complex transmits the light signal to the sensory system in the cytoplasm. In the present work, the truncated transducer, t-Htr was used which interacts with *p*pR [Sudo et al. Photochem. Photobiol. in press]. Two water-soluble reagents, hydroxylamine and azide reacted both to the transducer-free *p*pR and to the complex *p*pR/t-Htr (the complex between *p*pR and its truncated transducer). In the dark, the bleaching rates caused by hydroxylamine were not significantly changed between transducer-free *p*pR and *p*pR/t-Htr, or that of the free *p*pR was a little slower. Illumination accelerated the bleach rates because the reaction occurs selectively at the M-intermediate, but the rate of the complex was about 7.4-fold slower than that of the transducer-free *p*pR. Azide accelerated the M-decay, and its reaction rate of *p*pR/t-Htr was about 4.6-fold slower than the free *p*pR. These findings suggest that the transducer binding decreases the water accessibility around the chromophore at the M-intermediate. Its implication is discussed.

INTRODUCTION

The archaeon *Halobacterium salinarum* has four retinal proteins (archaeal rhodopsins), which are bacteriorhodopsin (bR) [1,2], halorhodopsin (hR) [3-5], sensory rhodopsin (sR, also called sensory rhodopsin I, sRI) [6-8] and phoborhodopsin (pR, also called sensory rhodopsin II, sRII) [9-11]. Former two, bR and hR are light-driven ion pumps for proton and chloride, respectively. The latter two, sR and pR are photoreceptors of this bacterium [9,11]. The ground state of sR (or sRI, the absorption maximum λmax of 587 nm) is a receptor mediating positive phototaxis, whereas its long-lived photo-intermediate (S373, λmax of 373 nm) acts as a receptor of negative phototaxis. pR (or sRII) absorbs maximally 487 nm light and works as a receptor of negative phototaxis [9]. Each receptor transmits its signals through integral membrane transducer proteins HtrI and HtrII [12-15] that are considered to form a signaling complex firmly with respective receptors. The Htr proteins both have a signaling domain and two methylatable domains [11,16,17] such as an aspartate receptor (Tar) in *Escherichia coli* [18], and their proteins activate a phosphotransfer cascade producing flagella motor responses. By these signaling systems, this bacterial cells move toward a longer wavelength light ($\lambda > 520$ nm) where bR and hR work, while they avoid a shorter wavelength light ($\lambda < 520$ nm) which contains harmful near-UV light.

It is reported that *Natronobacterium pharaonis* also have pR

(sRII)-like proteins [19-22]. The protein is called *pharaonis* phoborhodopsin (abbreviated as *p*pR; or *pharaonis* sRII, *p*sRII). *p*pR transmits its signal through transducer protein *p*HtrII as is the same as sR and pR. Recently, we [23] and Wegener *et al*. [24] succeeded in an expression of a truncated *p*HtrII (named as t-Htr) in *Escherichia coli*, where t-Htr is a N-terminal sequence of 159 amino acid residues of *p*HtrII. This t-Htr has the ability of exact contact with *p*pR, meaning that *p*pR/t-Htr (complex between *p*pR and t-Htr) serves as an adequate model system to elucidate the signal transfer. We [23] previously reported that the stoichiometry of *p*pR/t-Htr complex is 1:1. Functional unit of HtrI is considered to be a dimer [25]. If we accept this, the stoichiometry of *p*pR and *p*HtrII would be 2:2.

*p*HtrII-free *p*pR can transport protons on illumination [26,27]. On the other hand, this does not occur in the *p*pR/*p*HtrII complex [26,27] although the proton circulation (proton uptake and release occurs on the same side) is observed. We interpreted this as follows: at M-intermediate cytoplasmic channel (CP) of *p*pR may be closed by the association with *p*HtrII, whose idea was first proposed by Spudich *et al*. [8] for sR (sRI) system on the basis of the F-helix movement of bR. In the present paper, we attempt to obtain the evidence for the difference in conformational changes around the chromophore between transducer-free *p*pR and *p*pR/t-Htr.

Azide and hydroxylamine can react with the transducer-free *p*pR; 1) Azide accelerates greatly the decay of M-intermediate (ppR$_M$),

re-protonation process of Schiff base that forms between the chromophore and the ε-amino group of a lysine residue [28] and 2) hydroxylamine attacks the Schiff base mainly at *p*pR$_M$ state to bleach the pigment [29,30]. Since these two reagents are water-soluble, the increase in the both reactivity is considered to be caused mainly by the increase in the accessibility of these reagents to the hydrophobic moiety of the protein. Therefore, the reactivity of these reagents is a good indicator for the environmental change around the Schiff base. The present paper shows the decrease in the reactivity of both water-soluble reagents when the *p*pR/t-Htr complex forms, which supports the concept of the close of CP channel.

MATERIALS AND METHODS

Sample preparations

The expression of histidine-tagged recombinant *p*pR and t-Htr in *E. coli* BL21 (DE3), preparation of crude membranes and its purification were described previously [31,32]. The complex of *p*pR/t-Htr was prepared as previously described [23]. Sample media were exchanged by ultrafiltration (UK-50, Advantech, Tokyo, Japan) and finally samples were 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).

Reaction with hydroxylamine

Sample proteins were suspended in the buffer solution described above supplemented with 50 mM hydroxylamine. For the transducer-free *p*pR, the concentration of 5 μM was used. The complex of *p*pR/t-Htr was prepared by mixing 5 μM (final concn) of *p*pR and 50 μM (final concn) of t-Htr. The presence of 10-fold excess t-Htr was required for the complete complex-formation of *ppR*, details of which was described previously [23]. When necessary, samples were irradiated with a green light (with an interference filter of 506 nm, KL-50, Toshiba, Tokyo). A hot-mirror was placed in the front of the projector lamp (halogen-tungsten, 1 kW) to remove the heat radiation. The bleach process was monitored either by a spectrophotometer (V-560, JASCO, Tokyo) or by flash photolysis (see below). Experiments were done at 20 ˚C.

Reaction with hydroxylamine under pulse illumination

Preparation of the protein samples and the suspension medium were the same as those of the steady illumination. Pulse illumination was provided every 25 s with a Xe-flash lamp (duration of 250 μs) through an interference filter (KL-54, Toshiba, Tokyo) and a cut-off filter (Y52, Hoya, Tokyo). The bleaching was monitored by the absorbance at 500 nm.

Flash photolysis spectroscopy and calculation of the mean residence time (MRT)

Apparatus and procedure of the flash photolysis were essentially the same as described previously [33]. The M-decay was

monitored at 350 nm. The mean residence time of *p*pR_M, MRT was defined previously [29].

RESULTS

Figure 1 shows the absorption spectra of *p*pR/t-Htr complex at varying time after the constant illumination, and inset shows that obtained with the transducer-free *p*pR. The decreases in the absorbance at 500 nm and the concomitant increases in the absorbance at 360 nm were observed. The changes in these are proportional each other with an isosbestic point of 400 nm, implying that hydroxylamine can attack the Schiff base of *p*pR/t-Htr complex, as is similar to the transducer-free *p*pR (see inset).

The bleach by hydroxylamine in the dark was next examined. The amounts of unbleached protein were estimated by an amplitude of the flash-induced absorbance change. The ordinate of Fig. 2 represents the ratio of flash-induced absorbance changes after the incubation in the dark to that before the incubation. Open circles are the data of the transducer-free *p*pR and closed circles are those of the complex. The inset shows the semi-logarithmic plot, meaning that the reaction follows the first order kinetics. The bleaching rate of the transducer-free *p*pR was calculated to be 0.0044 min^{-1}, and that of the *p*pR/t-Htr was 0.0062 min^{-1}. Note that in the dark the rate of the transducer-free *p*pR is 1.4-fold slower than that of the complex.

Comparison of data shown in Fig. 1 and 2 leads to that the illumination accelerates greatly the bleaching rate, implying that photo-intermediate(s) has much higher reactivity than the ground state. In fact, we showed that hydroxylamine reacted selectively

with *p*pR$_M$ of the transducer-free *ppR* [29]. Since a photo-intermediate is attacked during the photocycling whose decay rates are different between the transducer-free *p*pR and the complex, the actual period for the reaction of hydroxylamine with an intermediate differs between the samples if the experimental condition of the steady illumination is employed. We, then, employed the pulse illumination as was used previously [29]. Results obtained are shown in Fig. 3, where the normalized amounts of unbleached *p*pR in the logarithmic scale are plotted against the number of flashes provided. This figure reveals the lower bleaching rate of the *p*pR/t-Htr complex (closed circles) than the transducer-free *p*pR (open circles). Although one flash evokes one chance of the reaction to the intermediate (*p*pR$_M$, see later), the lifetimes of *p*pR$_M$ are different and this correction must be necessary. Under the present condition, the M-decay time constants are 1.56 s^{-1} for the transducer-free *p*pR, and 0.90 s^{-1} for the *p*pR/t-Htr complex. It is noted that if the decay follows the single exponential equation, the mean lifetime of the intermediate or MRT (see later) is equal to the reciprocal of the decay constant. This correction led to that the bleaching rate of *p*pR decreases 7.5-fold by the association with its transducer.

Azide accelerates the decay of *p*pR$_M$ [28]. We measured *p*pR$_M$ decay rates of both the transducer-free *p*pR and *p*pR/t-Htr complex in the presence of varying concentrations of azide. The decay rate constants were calculated by a single exponential equation as was

previously employed [23]. Results are shown in Fig. 4. The both rate constants increased with an increase in the azide concentration. Under the azide concentration range examined, the rate increased linearly; when azide concentration is much larger than this range, the increase in the rate may level off as in [28]. The slope for the transducer-free *p*pR is 4.6-fold larger than that for the complex.

Previously we [29] showed that hydroxylamine reacts with M-intermediate of the transducer-free *p*pR. The experiments were done in the presence of varying concentrations of azide that accelerates the M-decay in the concentration-dependent manner but does not affect the L-decay (M-formation) neither the O-decay. Addition of azide, hence, modifies only the lifetime of *p*pR$_M$. The most convincing result was the strict proportional relationship between the bleaching rate and the mean residence time of *p*pR$_M$, MRT. Similarly, the rate of M-formation using the *p*pR/t-Htr complex was not changed (data not shown). We examined the relationship between the bleaching rate and MRT of M-intermediate under varying concentrations of azide. Results are shown in Fig. 5, revealing that *p*pR$_M$ of the *p*pR/t-Htr complex reacts with hydroxylamine, as is the same as the transducer-free *p*pR. In this figure, data of the transducer-free *p*pR were also plotted. The *p*pR/t-Htr complex obviously shows the high resistance against the hydroxylamine: The slope in the absence of t-Htr is 7.4-fold larger than that in the presence of t-Htr.

DISCUSSION

As shown in Fig. 2, hydroxylamine was able to attack the Schiff base of both the transducer-free *p*pR and the *p*pR/t-Htr complex in the dark, but their rates were very small. The bleach rate of the complex was a little faster (1.4-fold) than that of the free *p*pR. The amounts of unbleached pigments were estimated by the amplitude of flash photolysis. Illumination accelerated the bleach and the complex was more resistant against hydroxylamine under the illumination than the free *p*pR (see Fig. 3). Then, the difference of the bleach rate between the free and the complex might be a little larger than the 1.4-fold difference. This difference, on the other hand, means the complex formation of *p*pR/t-Htr in the dark.

In the dark, sR (or sRI) chromophore exhibits stability to hydroxylamine bleaching in the presence of HtrI. Removal of HtrI accelerates the bleaching rate by 2.4-fold [34]. On the other hand, for *p*pR, the presence of t-Htr increased the bleaching rate in the dark although the effect of t-Htr was small. The reason for the opposite tendency observed between *p*pR (*p*sRII) and sR (sRI) is not known at present, but maybe come from a structural difference in the ground state between sR/HtrI and *p*pR/t-Htr complex.

Illumination accelerates greatly the bleaching by hydroxylamine. Previously we [29] showed the ppR$_M$ selectively attacked by hydroxylamine using the transducer-free *p*pR. This paper shows that for the *p*pR/t-Htr complex, ppR$_M$ is also a selective target attacked by hydroxylamine (Fig. 5). We applied the

pulse-illumination instead of the steady illumination in order to avoid the complexity originated from the difference in the photocycling rate. After the correction of the lifetimes of *p*pR$_M$ of the transducer-free and associated *p*pR, we reached the conclusion that the association with the transducer decreases the reactivity of hydroxylamine by about 7.5-fold (Fig. 3). Results shown in Fig. 5 gave the same conclusion.

Subramaniam *et al*. [35] showed the L-intermediate of bR as the species that is reactive to hydroxylamine and this intermediate has protonated Schiff base. On the other hand, for *p*pR, M-intermediate is the species that has deprotonated Schiff base. In the dark, sR (sRI) chromophore is much easy to be bleached than bR [34], and their Schiff base is both protonated. Although the rates were very small, the rate of the wild-type *p*pR in the dark is one order larger than that of D75N under illumination [29]; both Schiff bases are protonated. These facts suggest that the reactivity of the Schiff base with hydroxylamine is influenced by the local environment of the chromophore. Because hydroxylamine is water-soluble, the susceptibility of this reagent might be reflected by the water accessibility around the chromophore. Therefore, we may conclude that association with the transducer reduces the water accessibility around the chromophore at *p*pR$_M$ state.

Recent reports on the structure of bR and its mutants [36-40] reveal the movement of helices during the photocycle. The

conformational change is largely located at the cytoplasmic end of helices F and G. These movements open a narrow water-accessible channel in the protein, enabling the transfer of a proton from an aspartate residue to the Schiff base. Similar helix movements during photocycle were also suggested in *p*pR by electron paramagnetic resonance (EPR) spectroscopy [24]. Under the condition that the transducer is present near *p*pR, the mobility of the spin label located at the side-chain of *p*pR decreases slightly. In other words, the photo-induced movement of helix F (or G) may be hampered by t-Htr.

Proton uptake occurred at the *p*pR$_M$ decay and the electrogenic proton transport from the cytoplasmic to the extracellular space was observed for the transducer-free *p*pR [26,27]. This is interpreted as: at *p*pR$_M$ or at its decay the cytoplasmic channel (CP) may open, leading to an increase in water accessibility to Schiff bases, which results in the increase in the reactivity. When *p*pR associates with the transducer, the electrogenic proton transport stops and only proton-circulation (uptake and release presumably at the extracellular (EC) channel) occurs [26]. This observation can be interpreted by the assumption of Spudich *et al*. [8] which is based on the helix tilt described above; the association of sRI or sRII (pR) with their respective transducer closes the CP channel, and the signal transmission is done by membrane helix-helix interaction that is induced by the movement of helix of the pigment. This closing CP channel may reduce the reactivity of hydroxylamine.

Mechanism of azide effect on the M-decay is proposed by two thoughts. One is that azide binds near D85 of bR (D75 of *p*pR) to create hydrogen networks in the CP channel [41], and the other is a shuttle mechanism through CP channel, details of which are described in [28,42]. When we adopt the shuttle mechanism, the association with the transducer makes the CP channel narrow, thus resulting in the decrease in the azide effect. Although the former is observed in bR and although no experimental data are available showing the validity for *p*pR, this mechanism leads to the conformational change in CP channel by association of t-Htr, which may also be accountable for the experimental findings. We could not rule out the possibility that amounts of the bound azide around D75 are larger for the transducer-free *p*pR. This might be less probable, however.

In the present paper, we showed that association of *p*pR with the transducer reduces the reactivity of *p*pR$_M$ to two water-soluble reagents of hydroxylamine and azide. These observations are consistent with the assumption that the transducer hampers the photo-induced outwardly helix movement of the pigment, which leads to the decrease in the reactivity with water-soluble reagents. This is consistent with the signal transduction mechanism proposed by Spudich [8,14].

ACKNOWLEDGMENTS

This work is partially supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

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FIGURE LEGENDS

Figure 1. Photo-induced difference spectra of the *p*pR/t-Htr complex and the transducer-free *p*pR (inset). Each spectrum (curve 1-3) was recorded at 2, 5 and 20 min after the addition of hydroxylamine, respectively. Illumination was provided with green light (506 nm at the intensity of 35 W/m^2). Proteins were suspended in 400 mM NaCl, 50 mM hydroxylamine, 0.1% DM and 10 mM Tris-HCl whose pH was adjusted at pH 7.0. Temperature was kept constant at 20 ˚C. Protein concentrations were 5 μM for the transducer-free *p*pR, and the complex of *p*pR/t-Htr was formed by adding 5 μM of *p*pR and 50 μM of t-Htr.

Figure 2. Reactivity of *p*pR to hydroxylamine in the presence and absence of t-Htr in the dark. Experimental conditions such as solution compositions, protein concentrations, hydroxylamine concentration and temperature were the same as those in Fig. 1. The unbleached amounts of *p*pR were estimated by flash photolysis. Open circles represent data of the transducer-free *p*pR and closed circles those of the *p*pR/t-Htr complex. Insets show the logarithmic plot.

Figure 3. Flash-induced bleach by hydroxylamine for the transducer-free *ppR* (open circles) and for the *p*pR/t-Htr complex (closed circles). The ordinate represents the normalized amounts

of unbleached (alive) pigment estimated by the amplitude of flash photolysis (at 500 nm) in the logarithmic scale, and the abscissa, the number of flashes. The experimental conditions were the same as in Fig. 1.

Figure 4. Azide accelerates the *p*pR$_M$ decay, but the effects differ between the transducer-free *p*pR (open circles) and the *p*pR/t-Htr complex (closed circles). Samples were 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) which was added by varying concentrations of azide. Temperature was 20 ˚C. The slopes are: 3.1 $s^{-1}mM^{-1}$ for the transducer-free *p*pR (open circles) and 0.68 $s^{-1}mM^{-1}$ for the *p*pR/t-Htr complex (closed circles).

Figure 5. Bleaching rates per one flash are plotted against the mean residence time of the M-intermediate, MRT. Open circles represent data of the transducer-free *p*pR and closed circles are those of the *p*pR/t-Htr complex. The calculation of MRT should be referred in [29]. The values of MRT were changed by adding various concentrations of azide. Bleaching rates per one flash were calculated from the slope of the plots similar to Fig. 3, and was corrected by subtracting the bleaching rate in the dark. It is noted that the bleaching rate in the dark could not be neglected because more than 100 min were required to achieve the measurement for each azide concentration. Experimental conditions were the same as in

Fig. 1 except for azide concentration.

Figure. 1

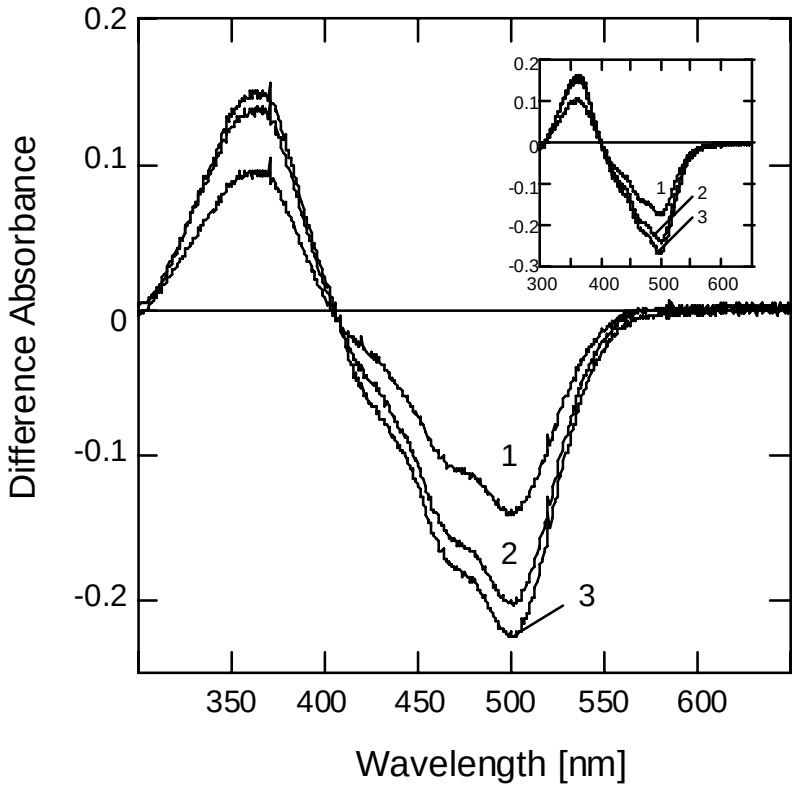

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Figure. 2

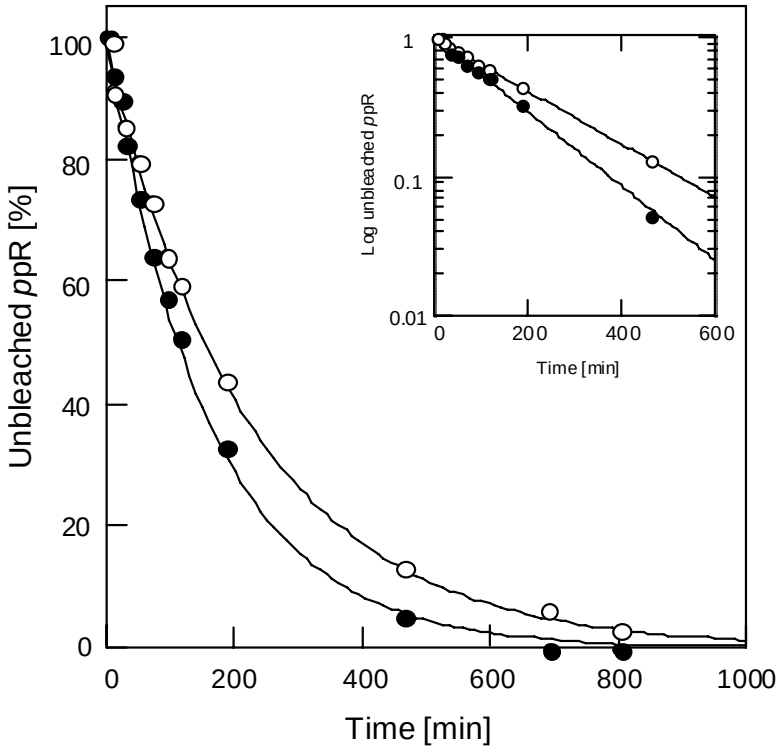

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Figure. 3

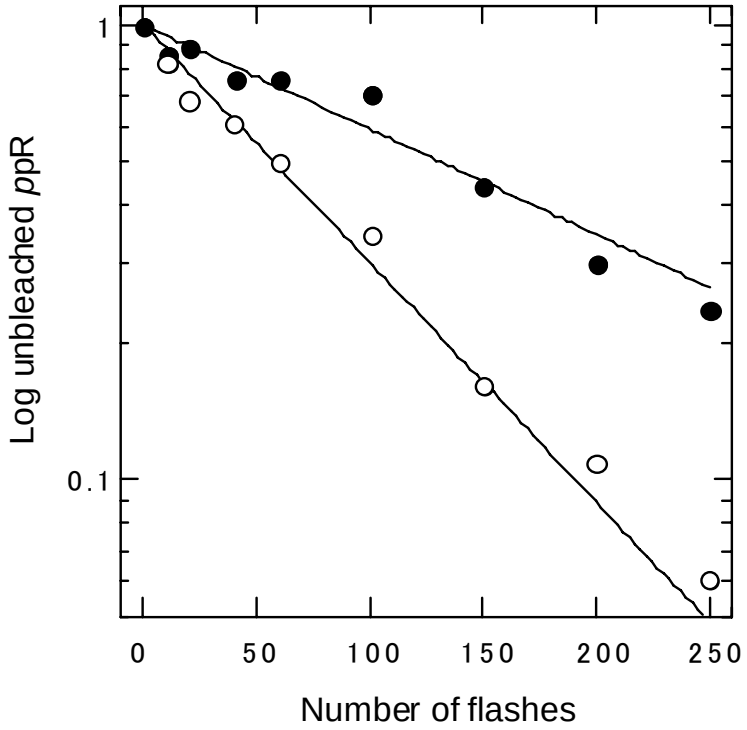

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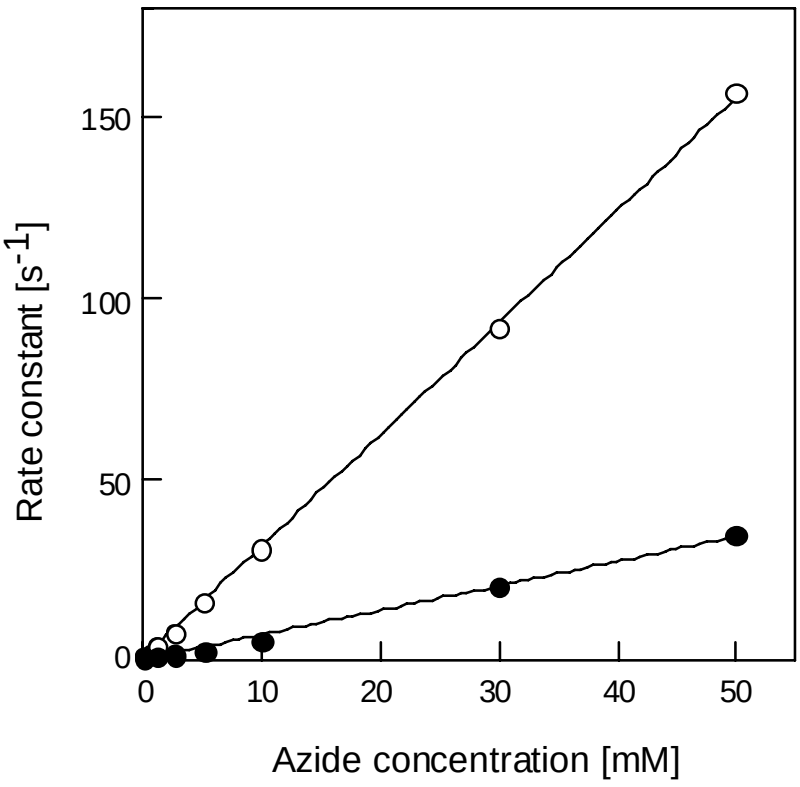
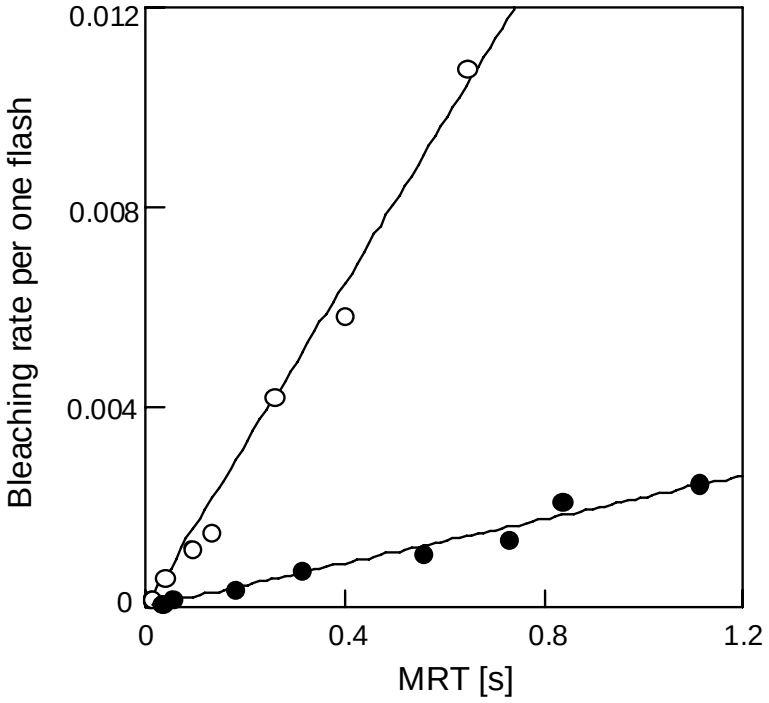
Figure. 4



Sudo *et al.*

Figure. 5



Sudo *et al.*