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Selective Reaction of Hydroxylamine with Chromophore during Photocycle of *pharaonis* Phoborhodopsin

by

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Running title: Bleaching of *pharaonis* phoborhodopsin

Abbreviations: Caps, N-cyclohexyl-3-aminopropanesulfonic acid; Ches, N-cyclohexyl-2-amino-ethanesulfonic acid; DM, n-dodecyl-ß-D-maltoside, D75N mutant, mutant in which Asp75 is substituted by Asn; Hepes, 2-[4-(2-hydroxylethyl)-1-piperazinyl]ethanesulfonic acid; Mes, 2-morpholinoethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; MRT, mean residence time; *p*pR, *pharaonis* phoborhodopsin; *p*pRM, M-intermediate of *p*pR; *p*pRO, O-intermediate of ppR.

ABSTRACT

Phoborhodopsin (pR; also called sensory rhodopsin II, sRII) is a receptor of negative phototaxis of *Halobacterium salinarum*, and *pharaonis* phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, psRII) is a corresponding protein of *Natronobacterium pharaonis*. These receptors contain retinal as a chromophore which bind to a lysine residue *via* Schiff base. This Schiff base can be cleaved with hydroxylamine to loose their color (bleaching). In dark, the bleaching rate of *p*pR was very slow whereas illumination accelerated considerably the bleaching rate. Addition of azide accelerated the decay of M-intermediate while its formation (decay of L-intermediate) is not affected. The bleaching rate of *p*pR under illumination was decreased by addition of azide. Essentially no reactivity with hydroxylamine under illumination was observed in the case of D75N mutant which lacks M-intermediate in its photocycle. Moreover, we provided illumination by flashes to *p*pR in the presence of varying concentrations of azide to measure the bleaching rate per one flash. A good correlation was obtained between the rate and the mean residence time, MRT which was calculated from flash photolysis data of the M-decay. These findings reveal that water-soluble hydroxylamine reacts selectively with M-intermediate and its implication was discussed.

INTRODUCTION

Halobacterium salinarum has at least four retinal proteins: bacteriorhodopsin (bR) [1-2], halorhodopsin (hR) [3-5], sensory rhodopsin (sR or sRI) [6-8] and phoborhodopsin (pR or sensory rhodopsin II, sRII) [9-11]. The former two work as light-driven ion-pumps and latter two as photoreceptors of this bacterium. sR (sRI) acts as a receptor of positive phototaxis and its long-lived photo-intermediate absorbing 373 nm maximally is a receptor of negative phototaxis. pR (sRII) is a photo-receptor of negative phototaxis whose maximum action locates at approximately 500 nm. Using these three photo-systems, this bacterium attracts to the longer wavelength light (> 520 nm) and avoids from the shorter wavelength light which contains harmful near UV light.

We [12-15] and Engelhard *et al*. [16-19] succeeded in the purification of a pR-like protein from *Natronobacterium pharaonis* and characterized the protein in great detail, because in a solubilized state it is much more stable than pR. We termed it *pharaonis* phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sRII). Recently, the functional expression of *p*pR in *Escherichia coli* was achieved [20], which provides large amounts of the protein and permits more detailed investigation.

Retinal as a chromophore binds to a lysine residue of these proteins *via* Schiff base. The Schiff base is susceptible to reaction with a water-soluble reagent, hydroxylamine [21], resulting in the bleaching of the pigment protein and producing retinal oximes. Bleaching of bR is enhanced by >2 orders of magnitude under

illumination [22], implying that hydroxylamine reacts with photo-intermediate(s). This enhanced reactivity was explained by the change of water accessibility accompanied with protein conformational change during photocycle. Thus, reactivity of Schiff base with hydroxylamine during photocycle is thought to be a good probe for the environmental change around Schiff base.

Reactivity of *p*pR chromophore with hydroxylamine was reported earlier [23], which focused on the retinal configuration of the ground state and intermediates. The present paper focused which intermediate is attacked by hydroxylamine to lead to the bleach. We concluded that hydroxylamine reacts with M-intermediate of *p*pR (*p*pR$_M$), which might suggest that distinct environmental change around chromophore of *p*pR occurs during photocycle and that the environment become hydrophilic at the M-intermediate.

MATERIALS AND METHODS

Sample preparations. The expression of histidine-tagged recombinant *p*pR in *E. coli* BL21(DE3) and its purification were described elsewhere [24]. The Kunkel method [25] was used to prepare D75N mutant.

Reaction of wild and D75N mutant of ppR with hydroxylamine under steady illumination. Hydroxylamine reactions were carried out at pH 7.0 in a mixture of six buffers (containing citric acid, Mes, Hepes, Mops, Ches and Caps, all concentrations of whose were 10 mM each), 0.1 % DM (n-dodecyl-ß-D-maltoside), 400 mM NaCl and 10 mM hydroxylamine. The mixture of six buffers has almost equal buffer capacity for whole pH ranges. Samples were irradiated with a green

light (with an interference filter of 506 nm, KL-50, Toshiba, Tokyo) from a 1-kW slide-projector lamp (Rikagaku Seiki, Tokyo). A hot-mirror was placed in front of the projector lamp to remove the heat radiation. Absorption spectra and time courses of absorbance changes at 500 or 520 nm were monitored by a spectrophotometer (V-560, JASCO, Tokyo).

Reaction of ppR with hydroxylamine under pulse illumination. Reactions were carried out under the same conditions as those of the steady illumination except for the hydroxylamine concentration of 50 mM. Samples were provided every 25 s by pulse illumination with a Xe-flash lamp (duration of 250 μs) through an interference filter (KL-50, Toshiba, Tokyo). The bleaching was monitored by the absorbance at 500 nm, and extent of bleaching was obtained as a function of the number of flashes.

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 [12]. The M-decay was monitored at 350 nm. The mean residence time of *p*pR$_M$, MRT was defined as

$$MRT = \int_0^\infty t\,C\,dt / \int_0^\infty C\,dt$$

where C represents the time-dependent flash-induced *p*pR$_M$ concentration of flash-photolysis data. Calculation of MRT was done numerically using Excel (Microsoft Japan, Tokyo) as follows: The

flash photolysis data were taken until *p*pR$_M$ disappeared completely and the number of data points acquired were 450. MRT was calculated by replacement of the integral with a summation;

$$MRT = \prod_{i=0}^{449} t_i C_i \delta t / \prod_{i=0}^{449} C_i \delta t = \prod_{i=0}^{449} t_i C_i / \prod_{i=0}^{449} C_i$$

where t_i and C_i are the time after the flash of i-th data point and the absorbance of at t_i, and δt is the period between successive data points.

RESULTS

Light-enhanced bleaching of wild-type ppR. In the dark, the bleaching reaction of the wild-type *p*pR proceeded very slowly (the rate of 0.0023 /min at 20 ˚C, data not shown) as is consistent with a previous paper [23], and illumination accelerated the bleaching. Figure 1 shows the change in the absorption spectrum of the wild-type *p*pR, where the hydroxylamine was 10 mM and pH was 7.0. The decrease in the absorbance at 500 nm and the concomitant increase in the absorbance at 360 nm was observed. These two are proportional with the isosbestic point of 400 nm, implying that the chromophore of *p*pR reacts with hydroxylamine and produces retinal oximes which has absorbance maximum at 360 nm. Using 33,600 $M^{-1}cm^{-1}$ of an extinction coefficient of retinal oxime [26], an extinction coefficient of *p*pR was calculated to be 42,000 $M^{-1}cm^{-1}$ which is very close to the value reported previously

[19]. Illumination is required for the prompt bleaching and the rate was 0.39 /min (see Fig. 3) which is about 170 times as fast as that in the dark, implying that hydroxylamine reacts with photo-intermediates of *p*pR.

The photocycle of *p*pR after ms time range is as follows: *p*pR $\rightarrow$ *p*pR$_M$ $\rightarrow$ *p*pR$_O$ $\rightarrow$ *p*pR where *p*pR$_O$ stands for O-intermediate. Addition of azide increased remarkably the M-decay rate while neither the M-formation and O-decay changed [27]. It is anticipated, therefore, that in the presence of azide, under the photo-steady state, the concentration of *p*pR$_M$ should decreases remarkably while that of *p*pR$_O$ should increase. The steady-state concentrations of *p*pR$_M$ and *p*pR$_O$ formed by illumination (506 nm) were measured in the presence or absence of azide (50 mM), and results were shown in Fig. 2. The concentration changes in *p*pR$_M$ and *p*pR$_O$ were monitored at 390 and 560 nm, respectively. At time 0 the continuous illumination was started to be provided, and in ten seconds the photo-steady state attained where the accumulation of *p*pR$_M$ and *p*pR$_O$ was observed. The absorbance decrease at 500 nm was due mainly to the decrease in the ground-state *p*pR, although absorptions of *p*pR and *p*pR$_O$ overlaps each other. Panel (a) shows data in the absence and (b) in the presence of 50 mM azide. As was expected, the photo-steady state concentration of *p*pR$_M$ decreased appreciably to almost zero in the presence of azide, while azide increased *p*pR$_O$ concentration.

Figure 3 shows the effect of azide on the bleaching rate under the continuous illumination (506 nm), where closed circles represent data in the absence of azide, and open circles represent data in

the presence of 50 mM azide. Unbleached (alive) concentrations of *p*pR were estimated from the absorbance at 500 nm and plotted in a logarithmic scale against the duration of the illumination. The bleaching reaction followed the first order kinetics and the rate constant of 0.39 /min was obtained in the absence of azide. Azide slowed down the bleaching rate to 0.027 /min. As is described above, hydroxylamine reacts with photo-intermediates, and azide induces the increase in the photo-steady state concentration of *p*pR$_O$ but the decrease in *p*pR$_M$ concentration. These findings suggest strongly that hydroxylamine reacts with *p*pR$_M$.

Asp75 of *p*pR which is the corresponding residue to Asp85 of bR [17] acts as a proton acceptor from the protonated Schiff base: At *p*pR$_M$, the Schiff base is de-protonated and the proton is transferred from the Schiff base to unprotonated Asp75 [18, 28]. D75N mutant, in which Asp75 was replaced by Asn, therefore, lacks the M-like intermediate and gives only one red-shifted photointermediate (O-like intermediate) in its photocycle in the ms time range [29]. We measured the reactivity of chromophore of D75N with hydroxylamine under the continuous illumination, and results are shown in Fig. 3 (open squares). In the case of D75N mutant, unbleached (alive) D75N was estimated from absorbance at 520 nm where its absorption maximum locates [29, 30]. The bleaching rate of this mutant was so slow to be 0.00034 /min, which is about 10th-fold slower than that of the wild-type in the dark even though D75N was illuminated. This finding on D75N yields additional evidence that *p*pR$_M$ reacts with hydroxylamine.

As shown in Fig. 2, the photo-steady state concentration of *p*pR$_M$ was almost zero in the presence of azide. On the other hand, the bleaching rate was slow but not zero (Fig. 3). This apparent discrepancy might come from the experimental condition of the continuous illumination; although the steady average concentration of *p*pR$_M$ is almost zero, every *p*pR molecule, under the continuous illumination, converts transiently to *p*pR$_M$ which can react with hydroxylamine. The continuous illumination was then changed to pulsed light. Results obtained were shown in Fig. 4, where the relative concentration of unbleached (alive) *p*pR monitored with 500 nm absorbance was plotted as a function of the number of flashes delivered to the wild-type *p*pR in the absence and presence of 50 mM azide. It is clear that greater number of photocycles (flashes) are required for the bleaching in the presence of azide than in the absence. We further investigated the relation between the bleaching rate and the existence probability of *p*pR$_M$ during a single photocycle turnover. For the estimation of the existence probability, mean residence time (MRT) was introduced which was defined in Materials and Method. We did the same experiments as Fig. 4 under varying concentrations of azide, and bleaching rate per one flash was evaluated. The period of successive flashes was employed to be 25 s because a singe photocycle completed about in 5 s. During this darkness between successive flashes, the reaction proceed in some extent. This correction was done. Although it was small, we could not neglect it for the data of high azide concentration. Results were depicted in Fig. 5 where both beaching rate per one flash and

MRT were plotted against the azide concentration. An inset reveals a good proportionality between MRT and the bleaching rate per one flash. This result gives the quantitative evidence that *p*pR_M reacts with hydroxylamine.

DISCUSSION

Water-soluble reagent hydroxylamine reacts with Schiff base of retinoid proteins and produces retinal oxime [21]. It was reported that the bleaching reaction is enhanced by >2 orders of magnitude under illumination in bR [22]. Therefore, hydroxylamine mainly react with photo-intermediate(s) in bR photocycle, reflecting the change of accessibility of hydroxylamine from outer milieu to the active center of the protein. Early work on bR attributed this light-enhanced reaction to the M-intermediate [31, 32], which is relatively long-lived and is known to be associated with deprotonation of the Schiff base and with substantial changes in the protein structure. However, this hypothesis was seriously questioned by Subramaniam *et al*. [22], who studied the reaction in a series of bR mutants solubilized in lipid/detergent micelles. No correlation was found between the efficiency of the light-induced reaction and the presence or absence (or the lifetime) of the M- and subsequent N- and/or O-intermediates. This led to the conclusion that the reaction occurs as a consequence of a conformational change caused by the light-catalyzed all-*trans*->13-*cis* isomerization, but before deprotonation of the Schiff base at the M-state. The L intermediate,

precursor of M was suggested as the species that is reactive to hydroxylamine.

On the other hand, this paper dealing with *p*pR showed that *p*pR$_M$ reacts with hydroxylamine. The possibility that *p*pR$_L$ (L-intermediate of *p*pR) is attacked by hydroxylamine like bR may be very small. The first reason is that azide influences mainly the *p*pR$_M$ decay in the ms to s time range of *p*pR photocycle [27]. Two thoughts have been proposed for the mechanism of this azide effect; one is that azide binds near D85 of bR and creates the hydrogen networks in the cytoplasmic channel [33], and the other is 'shuttle mechanism', details of which is described in [27,34]. Apart from the mechanism, azide affects mainly on the ppR$_M$ decay [27]. Therefore, it is very probable that the life-time (or MRT) of *p*pR$_M$ is influenced mostly by azide. The second reason is the quantitative test revealing the good correlation between MRT and bleaching rate per one flash (the inset of Fig. 5).

The L-intermediate of bR which is attacked by hydroxylamine has protonated Schiff base. *p*pR$_M$ has deprotonated Schiff base. It is reported that the ground state of sR (sRI) is bleached relatively easily than bR with hydroxylamine in the dark (N. Hazemoto, Ph.D. dissertation to Hokkaido University, 1983), and sRI has protonated Schiff base. 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; both of Schiff base are protonated. These facts suggest that the reactivity of the Schiff base is influenced greatly not by the protonated state of Schiff base but by the local environment

of the chromophore. Because hydroxylamine is water-soluble, the susceptibility of this reagent might be regulated by water accessibility around the chromophore.

Proton uptake occurs at the *p*pR$_M$ decay and an electrogenic proton transport from the cytoplasmic to the extracelluar space is observed [35]. This suggests that at the *p*pR$_M$ state the cytoplasmic channel of *p*pR may open, leading to the increase in water accessibility to Schiff base from outer milieu, which results in the increase in the reactivity with water-soluble hydroxylamine. Similar to bR [36-39], helix movement of *p*pR during photocycle was shown by electron paramagnetic resonance (EPR) spectroscopy [40]; an outward tilting of helix F is correlated with the early steps of the photocycle and sustained until the *p*pR$_O$ decays back to the ground state. Hence, the increase in the hydrophilicity described above might not be detected with the EPR technique. The reason for the low reactivity at *p*pR$_O$ is not known, but it might be related that the cytoplasmic channel might close after *p*pR$_O$ since during *p*pR$_O$ decay, proton release might occur through the extracellular channel [35].

The cytoplasmic channel of *p*pR is more hydrophobic than that of bR because carboxylic or polar residues such as Asp96 and Thr46 of bR are replaced by Phe and Leu in *p*pR, respectively [17]. Thus, it is expected that the reaction of hydroxylamine with *p*pR$_M$ might not be easy, but its long life might enhance the reactivity. Further experiments using cytoplasmic channel mutant like F86D/L40T mutant of *p*pR [41] would be interesting.

Spudich et al. [7] described that all data of both sRI and sRII

(pR) can be account for by the following assumption: the association of sRI with its transducer, HtrI closes the cytoplasmic channel, and the signal transmission is done by membrane helix-helix interaction which induced by the movement of helix of the pigment. If fact, Yan et al. showed that sRI-HtrI complex is more resistant to hydroxylamine in dark than transducer-free sRI [42]. If the open of the cytoplasmic channel of *p*pR (not complex) increases the reactivity of hydroxylamine, it is expected that the binding with the transducer (*p*HtrII) may reduce the reactivity because transducer may close the cytoplasmic channel [35]. Preliminary results show that *p*pR-tHtr (tHrt means truncated *p*HtrII, details of which see [40]) complex is more resistant to hydroxylamine under illumination but in dark, the reaction rates are almost the same for the complex and *p*pR alone. More precise experiments are now in progress.

Why hydroxylamine selectively reacts with *p*pR$_M$ while it reacts before the formation of M in the case of bR? It is difficult to answer this question but it may be related to the differences among two retinal proteins on the structural changes around the Schiff base during the photocycle. This discrepancy might be involved in the relation between structure and function of bacterial retinal proteins.

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Figure Legends

Figure 1. Spectral changes caused by the reaction with hydroxylamine under illumination. Each spectra (curve 1-6) were recorded at 0, 5, 10, 15, 20 and 30 min after addition of hydroxylamine (10 mM), respectively. Reaction mixture was irradiated with green light (506 nm at the intensity of 35 W/m^2. *p*pR of 5 μM was suspended in a buffer of pH 7.0 (whose composition was described in Materials and Method) at 20 ˚C.

Figure 2. Comparison of photo-steady state concentrations of photo-intermediates in the absence (a) and presence of 50 mM azide. Absorbance changes at 390 and 560 nm mainly monitor the accumulation of *p*pR_M and *p*pR_O, respectively, and those at 500 nm represent the decrease in the ground state pigment. Samples were started to be irradiated with green light (506 nm at the intensity of 35 W/m^2) at 0 sec of the abscissa. *p*pR of 5 μM was suspended in the buffer of pH 7.0, and temperature was 20 ˚C.

Figure 3. Bleaching kinetics of the wild-type and D75N mutant of *p*pR under the continuous illumination. Data shown by open circles show the relative concentration of unbleached (alive) wild-type *p*pR in the absence of azide, closed circles, wild-type ppR in the presence of 50 mM azide. Open squares show data of D75N in the absence of azide. Concentrations of unbleached (alive) pigment were estimated from the absorbance at 500 nm (for wild-type) and 520 nm (for D75N). The concentration of the pigments before illumination

was take as 1, and they were 5 μM for the wild-type and 3 μM for D75N. Here, an extinction coefficient was taken as 42,000 $M^{-1}cm^{-1}$ determined in text. Samples were irradiated with green light (506 nm at the intensity of 70 W/m^2). The buffer contained 10 mM hydroxylamine whose composition was described in Materials and Methods. Temperature was 20 ˚C.

Figure 4. Bleaching kinetics of the wild-type *p*pR in the absence (open circles) and presence (closed circles) of 50 mM azide under pulsed illumination. Samples were irradiated by flash light every 25 s and absorbance change at 500 nm were monitored. *p*pR of 5 μM was suspended in a buffer described in Materials and Methods and its concentration before illumination was taken as unity. Temperature was 20 ˚C.

Figure 5. Relationship between flash light-induced bleaching rate (open circles) and the mean residence time (MRT) of *p*pR_M (closed circles) under varying concentrations of azide. Bleaching rates per one flash were calculated from the slope of the plots similar to Fig. 4, and was corrected by subtracting the bleaching rate in the dark (flashes were not provided during measurement, data not shown). It is noted that bleaching rate in the dark cannot neglect because more than 100 min were required to achieve measurement of each azide concentration. Values of MRT were calculated from flash-photolysis data measured at 340 nm. *p*pR (5 μM) was suspended in a buffer described in text and pH was 7.0. Temperature was 20

˚C. *Inset*: a good proportional relation between MRT and bleaching rate per one flash.

Figure 1

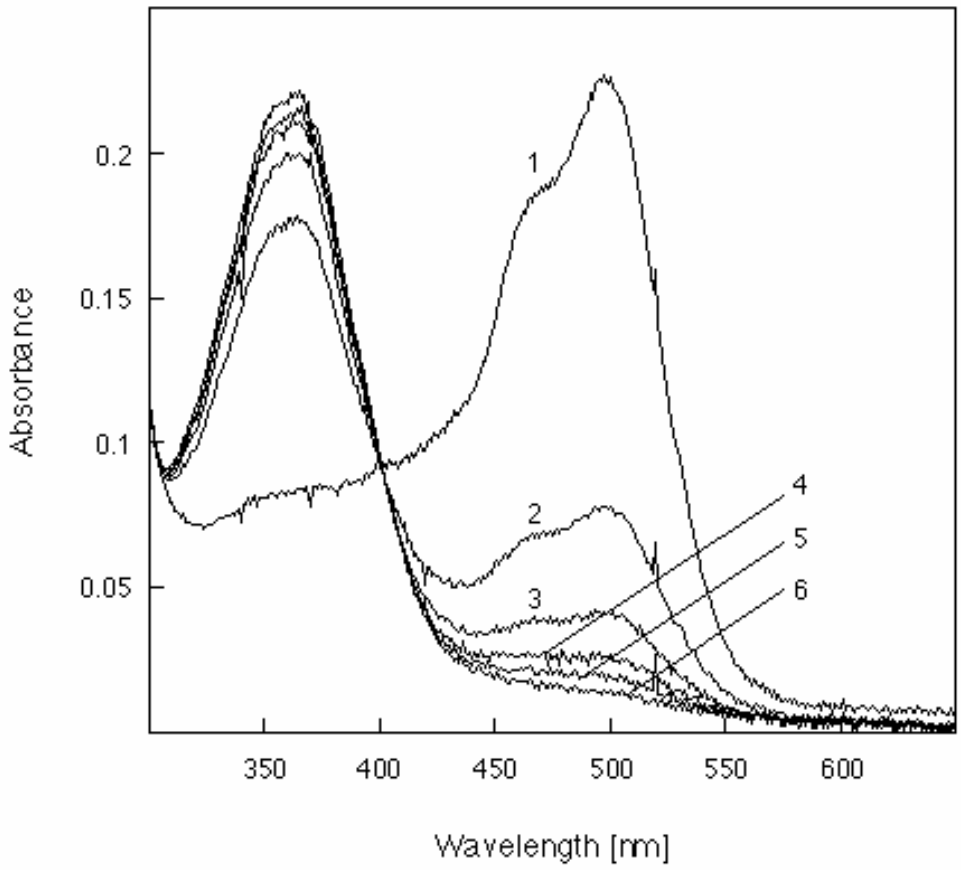

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Figure 2

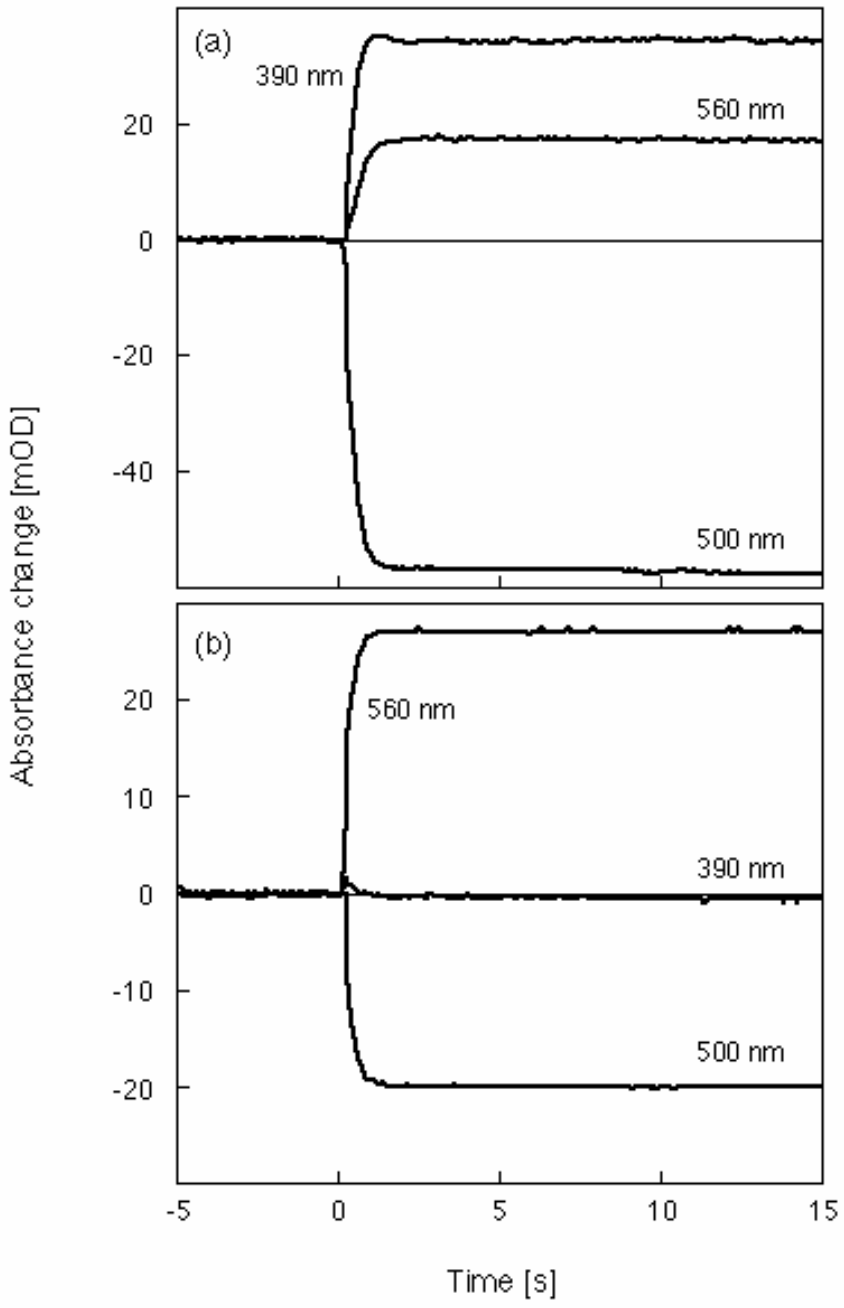

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Figure 3

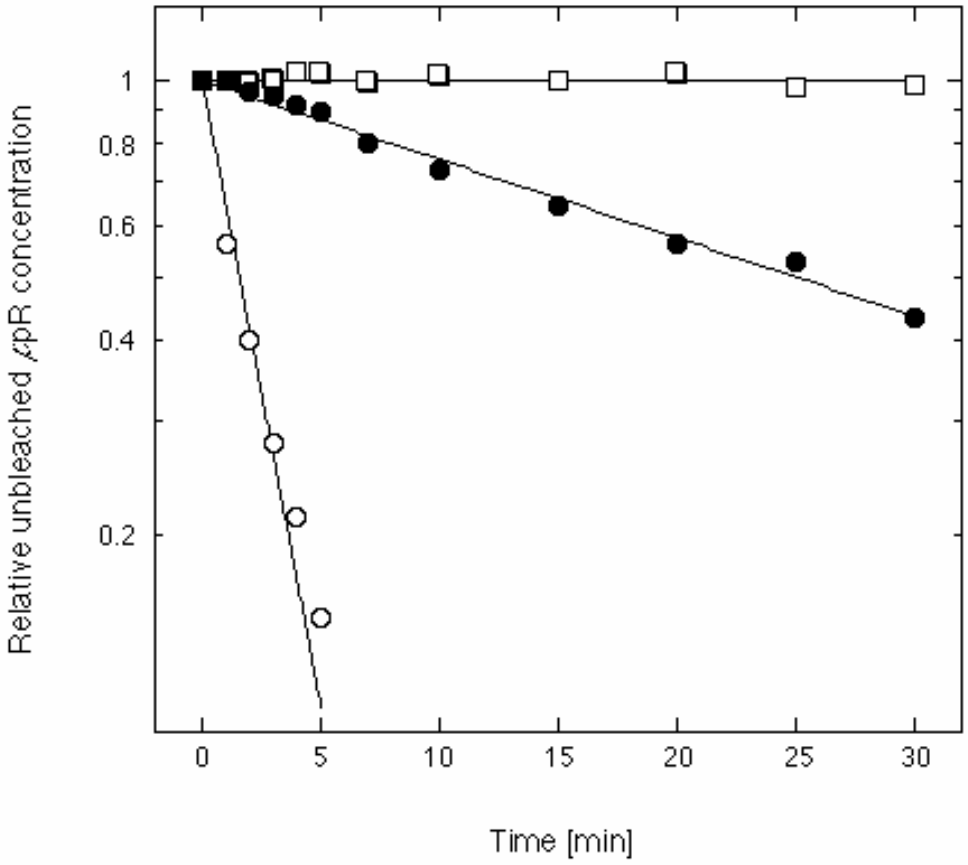

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Figure 4

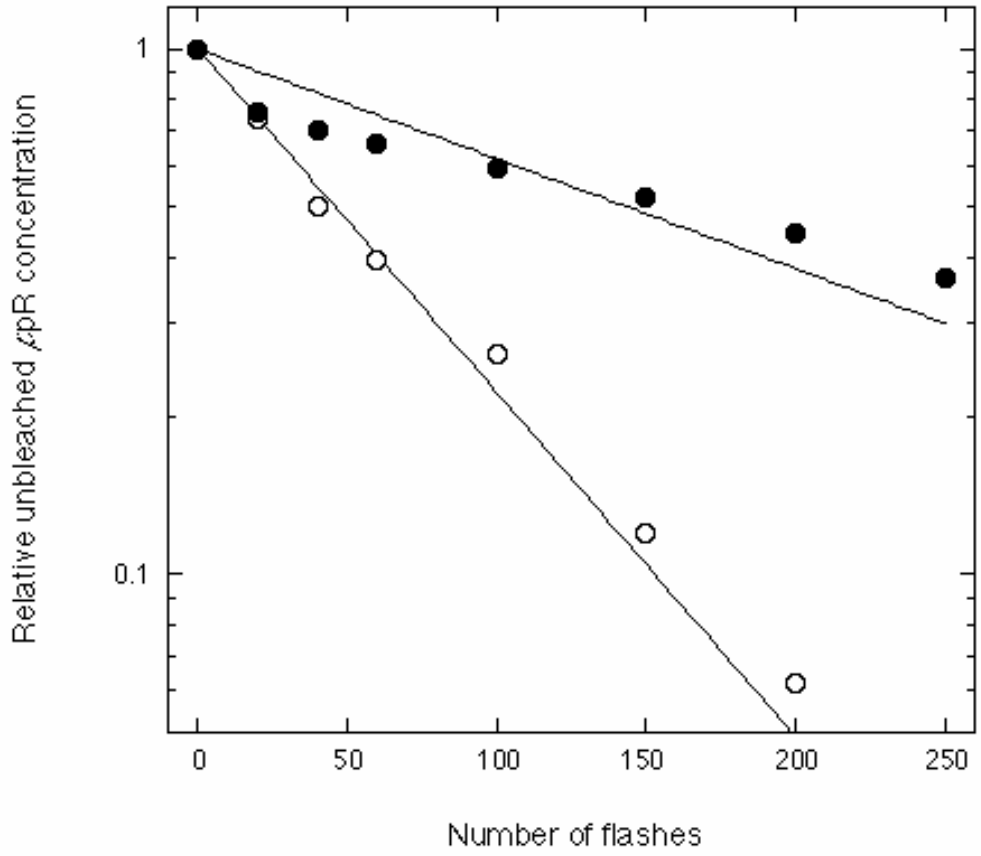

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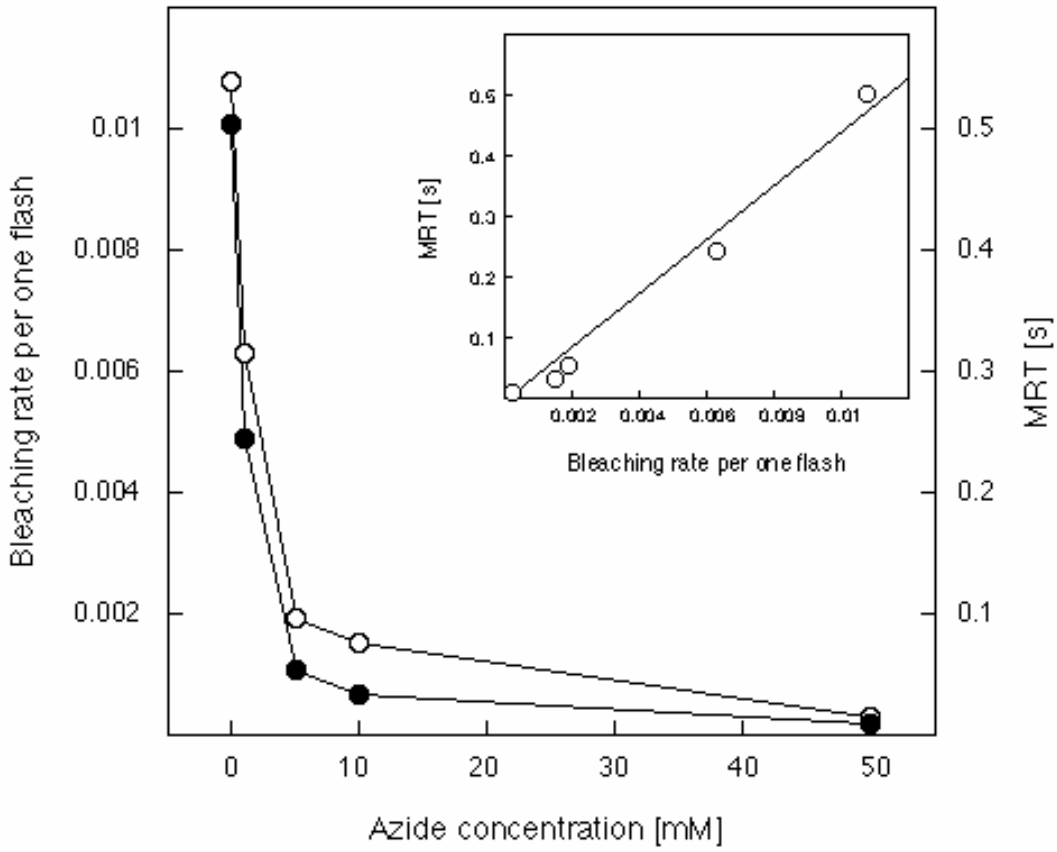
Figure 5



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