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Role of Arg-72 of *pharaonis* phoborhodopsin (sensory rhodopsin II) on its photochemistry

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[Running title] Arg-72 of *pharaonis* phoborhodopsin

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[Abbreviations] bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; pR, phoborhodopsin; *pp*R, *pharaonis*

phoborhodopsin (sensory rhodopsin II); NpsRII, *pharaonis* sensory rhodopsin II; *p*HtrII, halobacterial transducer for *pharaonis* phoborhodopsin (sensory rhodopsin II); Arg-82^{bR}, arginine residue at 82^{nd} position of bR; Arg-72^{ppR}, arginine residue at 72^{nd} position of *pp*R; EC, extracellular channel; CP, cytoplasmic channel; $R72A^{ppR}$, mutant in which Arg-72^{ppR} is replaced with Ala.

Summary

pharaonis phoborhodopsin (*p*pR, or *pharaonis* sensory rhodopsin, NpsRII) is a sensor for the negative photo-taxis of *Natronomonas (Natronobacterium) pharaonis*. Arginine-72 of *p*pR corresponds to Arg-82 of bacteriorhodopsin, which is a highly conserved residue among microbial rhodopsins. Using various Arg-72 *p*pR mutants, we obtained the following results: 1) Arg-72^{ppR} together possibly with Asp-193 influenced the pK_a of the counterion of the protonated Schiff base. 2) The M-rise became ca. 4-times faster than the wild-type. 3) Illumination causes proton uptake and release, and the pH-profiles of the sequence of these two proton movements were different between R72A mutant and the wild-type. It is inferred that Arg-72 connects the proton transfer events occurring at both the Schiff-base and an extracellular proton releasing residue (Asp-193). 4) The M-decays of Arg-72 mutants were faster (8~27 folds at pH 8 depending on mutants) than the wild-type, implying that the guanidinium prevents the proton transfer from the extracellular space to the deprotonated Schiff-base. 5) The proton pumping activities were decreased for mutants having increased M-decay rates, but the extent of the decrease was smaller than expected. The role of Arg-72 of *p*pR on the photochemistry was discussed.

Introduction

Halobacterium salinarum has four retinal membrane proteins: bacteriorhodopsin (bR)(Oesterhelt and Stoeckenius, 1971; Lanyi and Luecke, 2001; Haupts et al., 1999), halorhodopsin (hR)(Varo, 2000; Mukohata et al., 1999), sensory rhodopsin (sR or sRI) (Hoff et al., 1997) and phoborhodopsin (pR; also called sensory rhodopsin II, sRII)(Kamo et al., 2001; Sasaki and Spudich, 2000; Takahashi et al., 1985). The former two work as light-driven ion pumps; bR works as an outward proton pump while hR works as an inward Cl^- pump. The latter two work as photo-sensors that relay light signals to their cognate transducer proteins, which initiates a phosphorylation cascade that regulates the flagella motors in order to control phototaxis. The ground state of sRI (absorption maximum, λmax 587 nm) mediates positive phototaxis while its long-lived photointermediate (λmax 373 nm) is a sensor for negative phototaxis (Spudich and Bogomolni, 1984). Phoborhodopsin or sRII (λmax 480 nm) is also a sensor for negative phototaxis (Takahashi et al., 1985). Thus, this bacterium is attracted toward light longer than 520 nm where the two ion pumps can work (Tomioka et al. 1986), and repelled from the shorter wavelength light that contains harmful UV light. *pharaonis* phoborhodopisn (*pp*R) or *pharaonis* sensory rhodopsin II (NpsRII) is a photo-sensor in *Natronomonas (Natronobacterium) pharaonis* corresponding to pR (or sRII)

of *H. salinarum* (Hirayama et al., 1992; Lüttenberg et al., 1998). *pp*R (NpsRII) is more stable than pR (sRII), especially in dilute salt solutions (Scharf et al., 1992). Success in the functional expression of *pp*R in *Escherichia coli* cell membranes allowed a simple preparation of the protein in large amounts, which permitted more detailed investigations (Shimono et al., 1997).

The amino acid sequence has been determined for all four kinds of pigments, and the sequences aligned (Zhang et al., 1996; Shimono et al., 1998; Ihara et al., 1999). 3D-structures are now available for all archaeal rhodopsins with the exception of sRI: the structure of bR was reviewed by Lanyi and Luecke(2001) and Neutze et al. (2002), hR was by Kolbe et al.(2001), and NpsRII (or *pp*R) was by two groups, Luecke et al. (2001) and Royant et al (2001). The general features of the structure are quite similar with seven transmembrane helices (A to G) situated almost perpendicular to the membrane. The chromophore (all-*trans* retinal) binds to a lysine residue located on helix G via a protonated Schiff base. The structures of the membrane helices of *pp*R and bR are very similar to each other; the RMSD (root mean square deviation) value between bR and *pp*R is 0.95 $Å^2$ when the transmembrane helices are confined, and this value is reduced to 0.77 $Å^2$ with only C-G helices being confined (Pebay-Peyroula et al., 2002). On excitation by light, the chromophore of each of these four pigments undergoes an

all-*trans* → 13-*cis* isomerization (Imamoto et al.,1992a), which is followed by thermal relaxations to the original state through a set of photochemical intermediates (Miyazaki et al., 1992; Chizhov et al., 1998). This sequence is called photocycling. Differences between ion-pumping and photo-sensor rhodopsins are: the photocycling rate of the photo-sensor is much slow (~s) than that of the ion-pumps (~10 ms) (Imamoto et al., 1992b; Scharf et al., 1992), and the photo-sensor of sR and pR is associated with a cognate transducer within the membrane called HtrI and HtrII (*p*HtrII for *pp*R), respectively (Yao and Spudich, 1992; Hoff et al., 1997; Spudich, 1998; Zhang et al., 1999; Yang and Spudich, 2001). The X-ray structure of the complex between NpsRII (*pp*R) and its truncated *p*HtrII was solved by Gordeliy et al (2002).

Alignment of primary amino acid sequences identifies Arg-82^{bR} or Arg-72^{ppR} as a highly conserved residue among archaeal rhodopsins (Ihara et al., 1999; Spudich, 2000; Brown, 2001), and hence this residue is considered a very important residue. This Arg residue is also found in proteorhodopsin from sea bacteria (Beja et al., 2001), NOP-1 from *Neurospore crassa* (Bieszke et al., 1999), *Chlamydomonas reinhardtii* sensory rhodopsin (Sineshchekov et al., 2002) and Anabena sensory rhodopsins (Jung et al., 2003). The role of Arg-82^{bR} has been investigated in bR: this residue controls the proton

release to the extracellular space, the pK_a value of Asp-85^{bR} which is a counterion of the protonated Schiff base, and the rate of retinal thermal isomerization (Balashov et al., 1993; 1995; Govindjee et al., 1996).

The crystal structure of *pp*R (NpsRII) shows that the guanidinium group of Arg-72^{ppR} is oriented toward the extracellular side of the membrane compared to that of Arg-82^{bR} (Luecke et al., 2001; Royant et al., 2002): The distance of each guanidinium nitrogen atoms of Arg-72 from the Schiff base is ~11 Å whereas that of that in bR is ~8 Å. This difference is considered to cause the interaction between Arg-72^{ppR} and Asp-75^{ppR} as counter-ion of the protonated Schiff base to become weak in comparison with that in bR. This difference may give rise to pK_a differences of functionally important amino acids of *pp*R from those of bR.

The aim of this paper is to clarify the role of Arg-72^{ppR} on the photochemistry of *pp*R, and for this end, we investigated the photocycling and the proton transport of various Arg-72^{ppR} mutants. The M-decays of Arg-72^{ppR} mutants were faster (8~27 folds as pH 8 depending on mutants) than the wild-type, which is interpreted as that the positive charge of the guanidinium prevents the proton transfer through the extracellular channel (EC) to the unprotonated Schiff base during M-decay. In addition, this Arg-72^{ppR} controls the pK_a value of Asp-75^{ppR}, the counterion of the protonated Schiff base. The timing of the light-induced

proton release and uptake of Arg-72^{ppR} was found to be different from that of the wild-type. The preliminary results were presented at the 1st Asian Conference on Photobiology at Awaji Island (Ikeura et al., 2002).

Materials and Methods

Preparation of samples

Expression and purification of histidine-tagged recombinant *pp*R and Arg-72^{ppR} mutant proteins were essentially the same as described previously (Ikeura et al., 2003; Shimono et al., 1997). The proteins were reconstituted with L-•-phosphatidyl choline (PC from egg, Avanti, Alabaster, AL) with the molar ratio of 1:50, and the procedure was the same as described previously (Iwamoto et al., 2003; Kandori et al., 2001).

pH-titration

Proteins reconstituted with egg-PC were suspended in 400 mM NaCl supplemented with 6-mixed buffer (citric acid, MES, HEPES, MOPS, CHES and CAPS whose concentrations were 10 mM each). After washing 2 or 3 times (15,000 x g for 30 min) the sample was re-suspened. This 6-mixed buffer has the advantage of an approximately constant buffer strength over a wide pH range (Balashov et al., 1995). The spectra at varying pHs were obtained using U-3210 spectrophotometer (Hitachi, Tokyo Japan) in which an end-on photo-multiplier was installed to reduce the scattering artifact. The pH was

adjusted to the desired value using H_2SO_4 or NaOH. Temperature was 20 °C. Data were analyzed with a model of two interacting residues (Balashov et al., 1996) and data fitting was done using Microcal Origin software (Microcal Software, Morthampton, MA).

Flash photolysis measurements

For measurements in the time range longer than ms (such as M-decay, O-decay and the recovery of the original pigment), a Xe-flash (>540 nm, 200 •s of the duration) was used with an appropriate combination of filters (KL54/Y52, Toshiba, Tokyo Japan). The apparatus and methods were the same as those described previously (Miyazaki et al., 1992). For the measurement of M-rise, the second harmonic of the fundamental beam of the Q-switched ND-YAG laser (532 nm, 7 ns) was employed as an actinic light source, and data were accumulated 100 times for each run.

light-induced proton transfer (release or uptake)

The egg-PC reconstituted samples were washed 2 or 3 times and suspended in pure water. Fifty to one hundred microliters of this suspension (~10 μM of *pp*R) was dropped on a transparent SnO_2 electrode surface and dried (diameter of spot, ca. 10 mm). The adhesion was so strong that the protein was not detached from the electrode surface unless washed with a detergent. This has been inferred from the fact that repeated use of the electrode is possible without any change in the

signal amplitude. This SnO_2 electrode was used as a working electrode and another SnO_2 electrode was used as a counter electrode. A solution of 400 mM NaCl was sandwiched by these two electrodes, and the solution was supplemented with 1 mM of the 6-mixed buffer whose pH was adjusted with H_2SO_4 or NaOH to the desired value. An electronic circuit was used which was essentially the same as previous described (Iwamoto et al., 1999a). The light generated emf (electro-motive force) arisen between the two SnO_2 electrodes was picked up and amplified through a 15 Hz low-cut filter (MEG-1200, Nihon-Koden Co., Tokyo Japan). This filter eliminated signals due to the fluctuation of the baseline and differentiated the signals. The actinic light pulse (duration of 4 ms) was provided by a mechanical shutter as described elsewhere (Iwamoto et al., 2003).

light-induced proton transport

Photo-induced proton transport by the wild-type *pp*R or Arg-72^{ppR} mutants was assayed using inside-out membrane vesicles that were prepared by passing the cells through a French press (500 - 700 kg/cm^2, Ohtake Co, Tokyo Japan) (Ikeura et al., 2002; Sudo et al., 2001). The photo-induced pH change in the vesicle suspension was measured with a SnO_2 electrode. One hundred microliters of the vesicle suspension was confined on the surface of the SnO_2 electrode using a dialysis membrane. The amounts of *pp*R or mutants in this vesicle suspension were adjusted to be constant at 66 •g. The other

surface of this dialysis membrane is faced to 400 mM NaCl without buffer, with which another SnO_2 electrode as a counter electrode was contacted. Electric signals arising between a pair of SnO_2 electrodes were measured with a DC potentiometer (Potentiostat/Galvanostat 2000, Toho Tech. Res., Tokyo Japan). The constant actinic light was provided from 300 W Xe-lamp through a hot-mirror, an interference filter (500 nm) and a cut off filter (Y46, >460 nm).

Results

pK_a of the counterion of the Schiff base.

The pK_a of the counter-ion of the Schiff base, Asp-75^{ppR} can be estimated by pH-titration: when it is protonated, the maximum wavelength is red-shifted, and color turns to pink (Chizhov et al., 1998; Shimono et al., 2000). The titration data are plotted in Fig. 1, where the ordinate represents the ratio of A_{541} at the respective pH to that at a sufficiently low pH where all the pigment is in the pink form. This ratio represents the fraction of the pink form. This figure shows the order of the pK_a is that wild-type < R72K < R72A < R72Q, revealing that Arg-72^{ppR}, presumably due to the positive charge of guanidinium group, decreases the pKa of the counterion.

The titration curves were analyzed with the Henderson-Hasselbalch equation, and it was found that data were well fitted with an equation with a single pK_a for R72A^{ppR} and R72Q^{ppR} in which Arg-72^{ppR} was replaced with a neutral amino acid. In contrast, for the mutant containing a positively charged residue (R72K^{ppR}) as well as for the wild-type, the data cannot be fitted with a single pK_a equation but could be successfully fit using the schema described in an inset of Fig.1. This scheme implies that the pK_a of the counterion is affected by the ionization state of another residue (X) (compare pK_{a1} and pK_{a4}), and the pKa of X is, in turn, affected by the ionization state of Asp-75^{ppR}, the counterion (compare

pK_{a3} and pK_{a4}) (Balashov et al. 1996).

The pK_a values of wild-type determined here are different from those previously reported (Chizhov et al., 1998; Shimono et al., 2000), but our recent results (Mizuta et al., in preparation) show the large dependence on lipid species used.

The pK_a of the protonated Schiff base may be changed by the replacement of Arg-72^{ppR}. However, unfortunately the exact value cannot be determined, but seems to be a little larger than that of the wild-type in the dark (>12, Balashov in preparation).

Proton release or uptake at an earlier stage of the photocycling

During the photocycling of *pp*R, proton release and uptake occur (Iwamoto et al., 1999a). The upper left panel (A) in Fig. 2 shows light-induced proton transfer for the wild-type (400 mM NaCl, pH 5.0). Note that the signal has been differentiated with a 15-Hz low-cut filter. The positively going signal means proton release from *pp*R. This figure reveals that the proton release occurs first when illumination was applied, followed by the uptake. This observation is different from that reported previously (Iwamoto et al., 1999a). We note that the present sample is reconstituted with egg-PC, and that for solubilized *pp*R, proton uptake and release coincide with the O-formation and O-decay, respectively, as was reported previously (Iwamoto

et al., 1999a). R72Q^{ppR} shows first proton uptake followed by release as shown in panel B). The medium contained 400 mM NaCl at pH 8.0.

In lower two panels of Fig. 2, the magnitudes of the first events observed immediately after the light stimuli are plotted against the pH of the medium. The wild-type (panel C) and R72A^{ppR} (panel D) data are quite different: the mutant never shows positive values, meaning that proton uptake precedes release over the whole pH range examined. The results for R72Q^{ppR} were the same as R72A^{ppR} (data not shown). In contrast, the wild-type shows positive (pH of 3~7) and negative (pH of 7~10) signals. This figure clearly shows that the Arg-72^{ppR} affects the timing of the proton release, similar to the effected Arg-82^{bR} (Balashov et al., 1993; Govindjee et al., 1996).

Effect on the rise of M-intermediate

The rise of M-intermediate was measured after a laser flash: in the presence of 400 mM NaCl (pH=8.0), this process was analyzed well by a single exponential equation and the time constants observed were 0.05 and 0.22 $\bullet s^{-1}$ for the wild-type and R72A^{ppR}, respectively. The rate for the mutant is ca. 4 times larger than that of the wild-type. The disappearance of the positive charge of guanidinium facilitates proton transfer from the Schiff base to the deprotonated counter ion, Asp-75^{ppR}. Similar increases in the M-rise time constant

were observed for Arg-82 mutants of bR (ex. ca. 10-times increase of $R82A^{bR}$) (Balashov et al., 1993; Hatanaka et al., 1996).

Effect on the M-decay rate

Figure 3 shows the traces of flash-photolysis data (in 400 mM NaCl, pH 8.0) at selected wavelengths for the wild-type and various Arg-72 mutants of *pp*R. Clearly, Arg-72 mutants of *pp*R show much faster M-decay than the wild-type depending on mutants. The M-decay were analyzed by a single exponential equation, and the rate constants for various mutants of *pp*R were 1.7, 45.3, 14.3, 10.4 and 40.8 s^{-1} for the wild-type, R72A, R72K, R72Q and R72S, respectively. As a result of the fast M-decay, the O-intermediate shows a faster rise with a larger amplitude, but the decay rate of the O-intermediate or the recovery rate of the original pigment is scarcely changed.

R72A/D193N double mutant shows slow M-decay

Figure 4 shows flash photolysis data at typical wavelengths for the $R72A/D193N^{ppR}$ (left) and $R72A/D193E^{ppR}$ double mutants (right). It is very interesting that in contrast to the $R72A^{ppR}$ single mutant, $R72A/D193N^{ppR}$ shows that M-decay is about 2-times slower than that of the wild-type (Fig.3a). On the other hand, $R72A/D193E^{ppR}$ show very fast M-decay, which is the same as for the R72A single mutant.

M-decay rate of Mutants-mutants under varying pH

The M-decay rates of various Arg-72^{ppR} mutants of *pp*R were measured under varying pH. The results are plotted in Fig. 5 where the ordinate represents the logarithm of the M-decay rate constant, k_1 (s^{-1}). The mutants are R72A (■), R72K (◆), R72Q (▲), R72S (▼), R72A/D193N (□) and R72A/D193E (◪), and the data for the wild-type are shown by ○. This figure reveals that log k_1 is approximately linear with pH for all Arg-72^{ppR} mutants except for R72A/D193N and the wild-type. The slopes of these Arg mutants are 0.74 (R72A), 0.96 (R72K), 0.88 (R72Q), 0.58 (R72S) and 0.79 (R72A/D193E), indicating that the slope is close to unity except for R72S. For this mutant, relatively larger values are obtained in the pH range from 8.8 to 9.5; the reason for this is not known. If these are omitted for the regression, the slope will become larger.

In Fig.1, the pH-titration profile of R72K^{ppR} is different from that of the other Arg-mutants while the pH-profiles of mutants shown in Fig.5 are all similar. This may be because the lysine residue perhaps is in its neutral form under the condition of Fig.5 (pH 7.5-10) but bears a positive charge under the conditions of Fig.1 (pH <7).

Photo-induced proton pumping activity of Arg-72^{ppR} mutants

The process of M-decay involves the protonation of the deprotonated Schiff base. The proton-donating residue of bR, Asp-96 is replaced with Phe-86 in *pp*R (Seidel et al., 1995); the CP of *pp*R, then, is very hydrophobic, suggesting that the proton conductivity through the CP is very small. Nevertheless, light-induced proton pumping is observed for *pp*R alone, implying that proton transfer through CP occurs during M-decay. On the other hand, complex formation of *pp*R with *p*HtrII stops the light-induced proton pumping with no or only a small change in the M-decay rate (Sudo et al., 2001; Schmies et al., 2001; Hippler-Mreyen et al., 2003). This has been interpreted as follows: Association with *p*HtrII results in the closure of the CP channel, and hence, for the complex, the proton should come to the deprotonated Schiff base solely through EC, implying no proton pumping activity of the complex. For *pp*R alone, the ratio of the proton conductivity of EC to CP might be large and then the M-decay rate of *pp*R might be unchanged by its association with *p*HtrII in spite of the CP-channel closure. The proton is released to EC and taken up both from EC and CP during photocycling. Therefore, the proton uptake via EC is futile for the photo-induced proton pumping of *pp*R, and the photo-induced proton pumping activity of *pp*R alone is a rough measure for the fraction of the proton entry from CP to the deprotonated Schiff base.

Results of light-induced proton pumping experiments with inside-out membrane vesicles containing various Arg-72^{ppR}

mutants are shown in Fig.6. The amount of pigment was kept constant for all mutants (66 μg). No light-induced proton movements were observed for the membrane vesicles derived from cells containing the vector alone, which did not express *pp*R (data not shown). In Fig. 6, two corrections have been made to account for two distortions of the data: The first correction is for the fraction of the bathochromic pink form that is unable to pump protons due to the protonation of the counterion of Schiff base, Asp-75^{ppR} in the dark. This fraction was estimated from the pK_as shown in Fig.1. The second correction is for the turn-over rate: the amounts of protons transported per unit time should be proportional to the rate, which was estimated from the half-time of the recovery to the original pigment. The activity of R72A/D193N^{ppR} is almost equal to that of the wild-type (exactly speaking, ~80%), and R72A^{ppR} and R72A/D193E^{ppR} show about 30% of the activity of the wild-type. It is interesting that the mutants having the faster M-decay have lower activity than those having the slower M-decay.

Discussion

Assignment of the residue X

Results of Fig.1 reveal that the positive charge at the Arg-72^{ppR} position decreases the pK_a of Asp-75^{ppR}, the counter-ion of the Schiff base. Similar observations have been reported for bR. The pK_a of the counter-ion for R82A^{bR} and R82Q^{bR} was increased to ca. 7 while that of the wild-type was 2.5, and the replacement by the positively charged residue (R82K^{bR}) kept the pK_a at a relatively low value of 3.5 (Balashov et al., 1995). The magnitudes of pK_a changes in *pp*R caused by the replacement with the neutral amino acids are smaller than those of bR, however. This may be because of the difference in the direction of the guanidinium pointing toward the Schiff base in bR or pointing away from the Schiff base in *pp*R, which increases the distance between the positive charge and the counterion in bR (Luecke et al., 2001; Royant et al., 2001).

As described in Results, the residue X is required for quantitative explanations for Fig. 1 (see the inset). What is the residue X? Since Asp-75^{ppR} is deprotonated in the dark at neutral pH, the pK_a of the residue X may be 5.7, that is the pK_{a2} value of the wild-type. The residue X might be Asp-193^{ppR}. Reasons for this assignment are; it has been shown that pK_a of the counter-ion (Asp-85^{bR}) in bR is affected by the protonation state of Glu-204^{bR} (Richter et al., 1996; Govindjee et al., 1996) that corresponds to Asp-193^{ppR}. In

addition, we showed previously that the electric charge of residue 193 of *pp*R affects the pK_a of the Schiff base (Iwamoto et al., 2002a), indicating that Asp-75^{ppR}, Arg-72^{ppR} and Asp-193^{ppR} are connected via hydrogen bonding through water molecules as is shown in X-ray crystal structure (Pebay-Peyroula et al., 2002).

light-induced proton transfer reactions on the wild-type and Arg-72^{ppR}.

Figure 2 shows the difference between the order of the flash-induced proton release and uptake between the wild-type and Arg-72^{ppR}. Iwamoto et al. (in preparation) showed that light-induced proton release at the earlier stage of the photocycling was not observed for D193N^{ppR} under the present conditions, suggesting strongly that Asp-193^{ppR} is a key residue involved in proton-release. Note that Asp-193^{ppR} corresponds to Glu-204^{bR} that is one of members of the proton-releasing complex in bR (Balashov et al., 1997; Dioumaev et al., 1998; Koyama et al., 1998).

In bR, the movement of the orientation of the side-chain of Arg-82^{bR} during photocycling is considered to cause a pK_a change of the proton-releasing complex consisting of Glu-194^{bR} and Glu-204^{bR} (Tanio et al., 1999; Petkova et al., 1999; Royant et al., 2000; Luecke et al., 1999, 2000; Neutze et al., 2002). Therefore, the pK_a change in a group analogous to Glu-204^{bR}, the proton-releasing Asp-193^{ppR} and the role of

Arg-72^{ppR} in wild-type *pp*R are very interesting. So far we have no information concerning the possible movement of Arg-72^{ppR} during photocycle.

At the pH where the first proton release is observed from the wild-type, Asp-193^{ppR} may be protonated, and then proton transfer from the Schiff-base to deprotonated Asp-75^{ppR} leads to the proton release from the protonated Asp-193^{ppR}. A proton absorbed after release is used for the protonation of the Schiff-base and the deprotonated Asp-193^{ppR} is reprotonated with the proton from Asp-75^{ppR} during O-decay. Above pH 7, Asp-193^{ppR} may be deprotonated, presumably for the earlier intermediates and certainly at the *pp*R ground state (pKa ~ 5.7) so that the first proton release cannot be induced. The decrease in the signal (its absolute magnitude) above pH 9 might be caused by the slowing-down of the proton-uptake rate due to high pH in the medium. Below pH 4.5, why does the proton-uptake first occur even though Asp-193^{ppR} may be protonated? The proton concentration in the external medium might be so high that the proton cannot be released from Asp-193^{ppR}, resulting in the first proton uptake followed by the slow release.

In contrast to the wild-type, R72A^{ppR} shows first light-induced proton-uptake over the whole pH range, followed by release. Thus, for the Arg-mutant, it seems that the proton transfer from the Schiff-base to Asp-75^{ppR} cannot be 'transmitted' to Asp-193^{ppR}, a proton-releasing residue,

due to the lack of the arginine residue. The proton now on Asp-~~75~~193^{ppR} may be used for the reprotonation of the Schiff-base; if so, proton which is released may be originate from the deprotonation of Asp-75^{ppR}.

M-decay of Arg-72^{ppR}

A remarkable observation from Fig.3 is that the M-decay of Arg-72^{ppR} mutants is faster than that of the wild-type. As postulated previously (Sudo et al., 2001), the proton that reprotonates the deprotonated Schiff base at the M-decay comes from both the CP and EC side, and due to the hydrophobic nature of CP, the main route may be the one through the EC domain. Therefore, we conclude that Arg-72^{ppR}, located in EC, hinders proton transfer through the EC to the deprotonated Schiff base at M-decay. This in turn prolongs the lifetime of the M-intermediate of the wild-type, one of signaling states for phototaxis (Yan et al., 1991).

Figure 4 shows the slow M-decay of R72A/D193N^{ppR} in sharp contrast to those of R72A/D193E^{ppR} and the Arg-72^{ppR} single mutants (Fig.3). This result implies that for R72A^{ppR} and presumably all Arg-72^{ppR} mutants, proton uptake from the EC side is mediated or controlled by Asp-193^{ppR} that is located nearly at the end of EC, open to the external medium. We note that log k_1 is approximately a linear function of pH in the region of 7.5 ~ 9. This may be interpreted as the passive proton transport through EC of Arg-72^{ppR}. Another possible

interpretation is that the proton transfer at M-decay of Arg-72^{ppR} is mediated by the protonated Asp-193^{ppR}. In this pH range, the Henderson-Hasselbalch equation predicts that the slope should be minus one when the logarithm of the fraction of protonated carboxyl group is plotted against pH, because the pK_a of Asp-193^{ppR} is probable far lower than the pH investigated (5.7 at the *pp*R ground state and pK_a for the intermediates are not known). A further interesting problem is whether Asp-193 of the wild-type *pp*R also controls the proton transfer through EC. Studies are now in progress using D193N^{ppR}.

Proton pumping activity

Figure 6 shows that the mutants having faster M-decay have lower light-induced proton pumping activity compared to the wild-type. If we accept the concept that the increase in the M-decay rate of Arg-72^{ppR} mutants is due to the increase in the proton transfer through EC and that this proton pathway does not contribute the proton-pumping, the results of Fig.6 are qualitatively conceivable. At pH = 8, the M-decay rate constant of the wild-type is 1.7 s^{-1} while that of R72A^{ppR} is 45.3 s^{-1}, indicating that in the mutant almost all protons which cause the reprotonation of the Schiff base during the M-decay are from the EC. This would predict that the proton pumping activity might be more reduced than that observe. Therefore, we would have to consider other factors that

control the light-induced proton pumping activity. One possibility is a 'two photon process' which was hypothesized originally to account for the unexpected strong proton pumping of the wild-type in the presence of azide (Schmies et al., 2000), in which the O-intermediate was accumulated due to the fast M-decay, similar to the present experiment. The photo-reactivity of the O-intermediate increases the turnover rate of the pigment under continuous illumination. Iwamoto et al. (2002b) verified the photo-reactivity. A second possibility is an increase in the proton conductivity in the CP caused by the mutation of the arginine residue even though it is located in EC. The last possibility is that there might be two M-states as is in bR (Lanyi and Schobert, 2003), and one only contributes to the proton pumping (M2 as is in bR). We should take consideration of the transition from M1 to M2, or population of these two M being affected by the mutation. The spectroscopic separated two-M of the wild-type *p*pR was described (Chizhov et al., 1998; Rivas et al., 2003). Further quantitative study on this respect should be necessary.

Effect on the chloride concentration in the medium

The Cl^- effect on characteristics and photochemistry of *p*pR has been reported (Iwamoto et al., 2002a; Shimono et al., 2000). In addition, we observed here a Cl^- effect on M-decay rate of $R72A^{ppR}$ (Fig.7). As discussed above, this result may

indicate a Cl^--effect on the pK_a of Asp-193^{ppR} of R72A. The Cl^- concentration we used here was only 400 mM NaCl.

Concluding remarks

In the present paper, we conclude the following: 1) Arg-72^{ppR} decreases the pK_a of Asp-75^{ppR}, the counterion of the protonated Schiff base. 2) Replacement of Arg-72^{ppR} increases the M-decay rate, which may be the result of increased proton transfer rate through the EC. In other words, Arg-72^{ppR} in the wild-type prevents this proton transfer, leading to the prolongation of the life time of M-intermediate; the effect is enhanced by the lacking of a proton donating residue at the Asp-96^{bR} position (Iwamoto et al., 1999b). 3) Substitution for the arginine residue at position 72 changes the timing of proton release and uptake from that of the wild-type. The lack of the arginine residue may disconnect the intra-molecular proton transfer chain from the Schiff-base and the extracellular part. Further investigation on the role of Arg-72^{ppR} on proton transfer will be of interest. One reason may be because the orientation of the guanidinium in the dark is opposite to that of bR; we do not know yet whether this guanidinium group of Arg-72 moves to change the distance from Asp-193 during photocycling as bR.

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Figure Legends

Figure 1. Spectroscopic pH titration to determine pKa of Asp-75^{ppR}, the counterion of the protonated Schiff base. The ordinate represents the ratio of the 541 nm absorbance at the respective pH (see text). The protein (ca. 10 μM) was suspended in 400 mM NaCl supplemented with 6-mixed buffer (see Materials and Methods section), and the pH was adjusted with H_2SO_4 or NaOH. The titration data were analyzed with the scheme shown in the inset and pK_a values were estimated with a non-linear regression. Temperature was 20 °C.

Figure 2. Flash-induced proton transfer reaction of the wild-type (panel A and C) and Arg-72^{ppR} mutant (panel B and D). R72Q was used in panel B and R72A was used in panel D. Panel A and B show the proton transfer data measured by SnO_2 electrode. The potential difference between a pair of SnO_2 electrodes was measured using a 15 Hz low-cut filter, which gave the differentiated signals. For the wild-type (panel A), the light pulse (4 ms duration) led first to proton release, followed by the uptake. Panel B shows the opposite sequence for R72Q^{ppR}. The magnitudes of the first photo responses are plotted against pH in panels C) and D) for the wild-type and R72A^{ppR}. The medium for all experiments was 400 mM NaCl. The pH values in panel A) and B) were 5.0 and 8.0, respectively. For detailed experimental procedures see the Materials and

Methods section.

Figure 3. Flash photolysis data of selected wavelengths for the wild-type (A), R72A^{ppR} (B), R72K^{ppR} (C), R72Q^{ppR} (D) and R72S^{ppR} (E). All samples were reconstituted with egg-PC. Absorbance changes in 390, 500 and 560 nm monitor the concentration of M-intermediate, *pp*R and O-intermediate, respectively. The medium was 400 mM NaCl buffered with 10 mM of MES plus CHES at pH 8.0. Temperature was 20 °C.

Figure 4. Flash photolysis data of selected wavelengths for R72A/D193N (left) and R72A/D193E (right) *pp*R mutants. Experimental conditions were the same as in Fig.3.

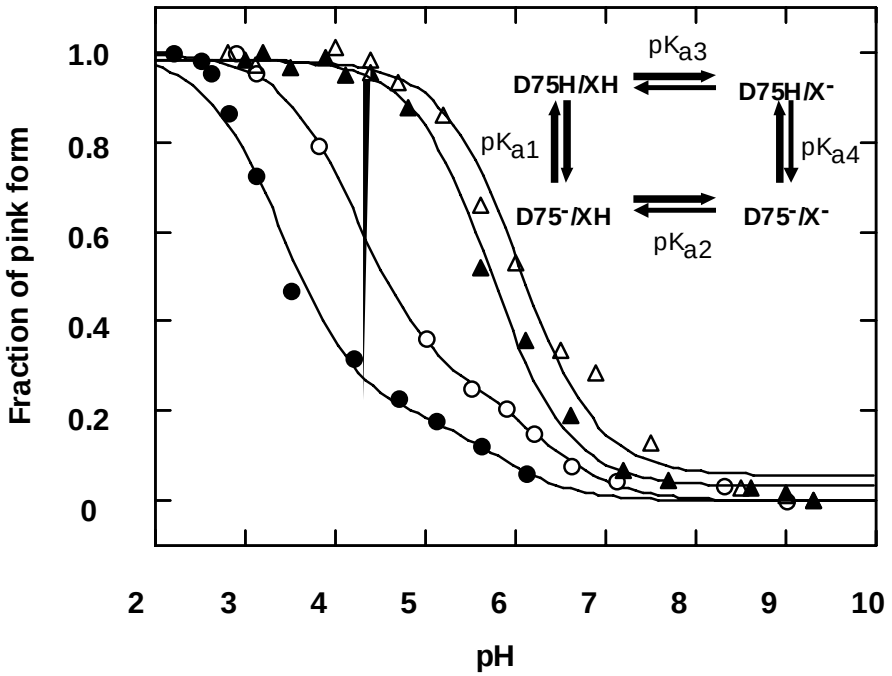
Figure 5. The logarithm of k1, the rate constant of the M-decay is plotted against pH in the medium. The mutants are R72A (■), R72K (◆), R72Q (▲), R72S (▼), R72A/D193N (□) and R72A/D193E (◪), and data of the wild-type are shown by ○. Experimental conditions were the same as in Fig.3.

Figure 6. Photo-induced proton pumping through inside-out membrane vesicles derived from *E. coli* cells expressing *pp*R or mutants. Data were corrected by two factors (for details, see Results section). ●, the wild-type; ○, R72A;

▲,R72A/D193E; △, R72A/D193N. The medium contained 400 mM NaCl at pH 6.5 without buffer.

Figure 7. Cl^- concentration dependence of the M-decay rate constant of $R72A^{ppR}$. The closed circle shows the data in the presence of 400 mM NaCl and the open circle shows the data in the absence of Cl^-. The medium was buffered with 10 mM MES plus CHES at pH 8.

Figure 1 (Ikeura et al.)



		pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
●	Wild-type	3.5	5.7	4.1	5.1
▲	R72A	5.7			
○	R72K	4.4	6.2	4.8	5.7
△	R72Q	6.0			

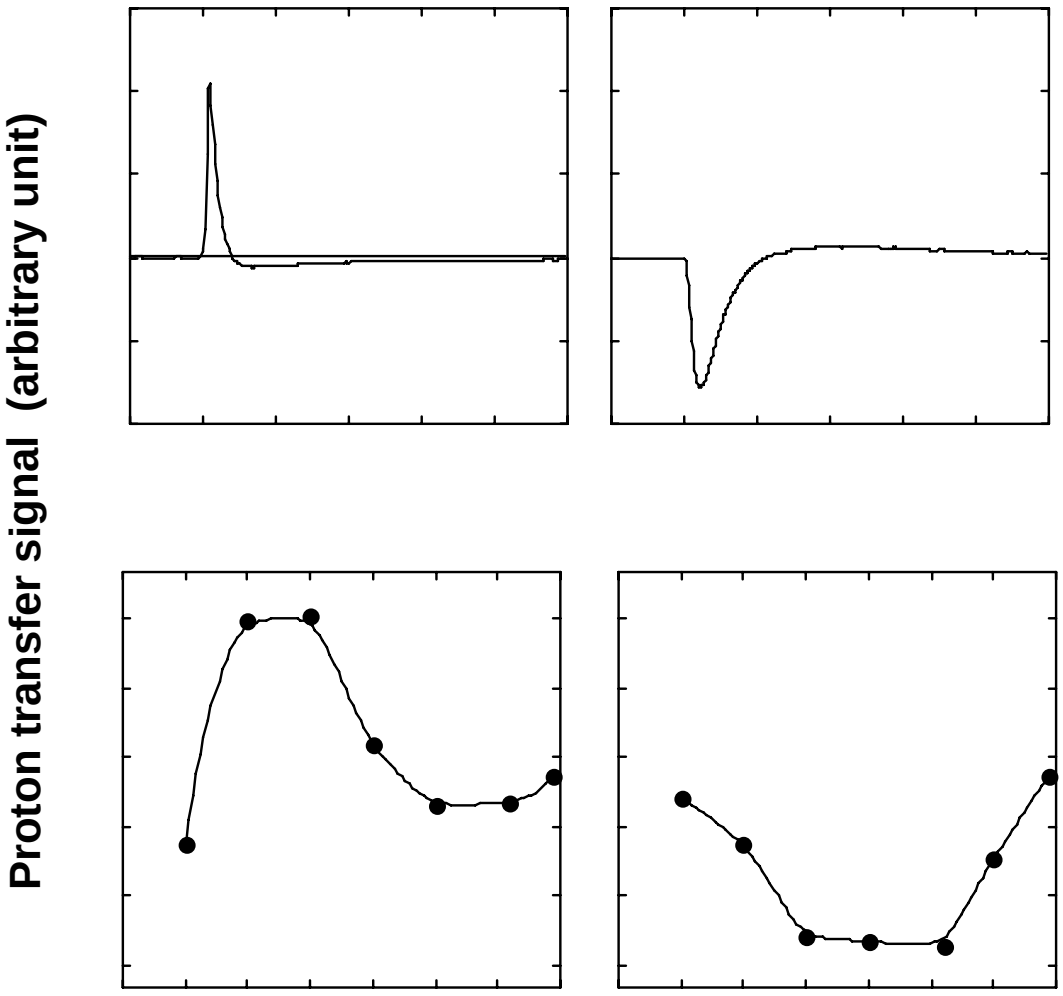

Proton transfer signal (arbitrary unit)

Fig.3 (Ikeura et al.)

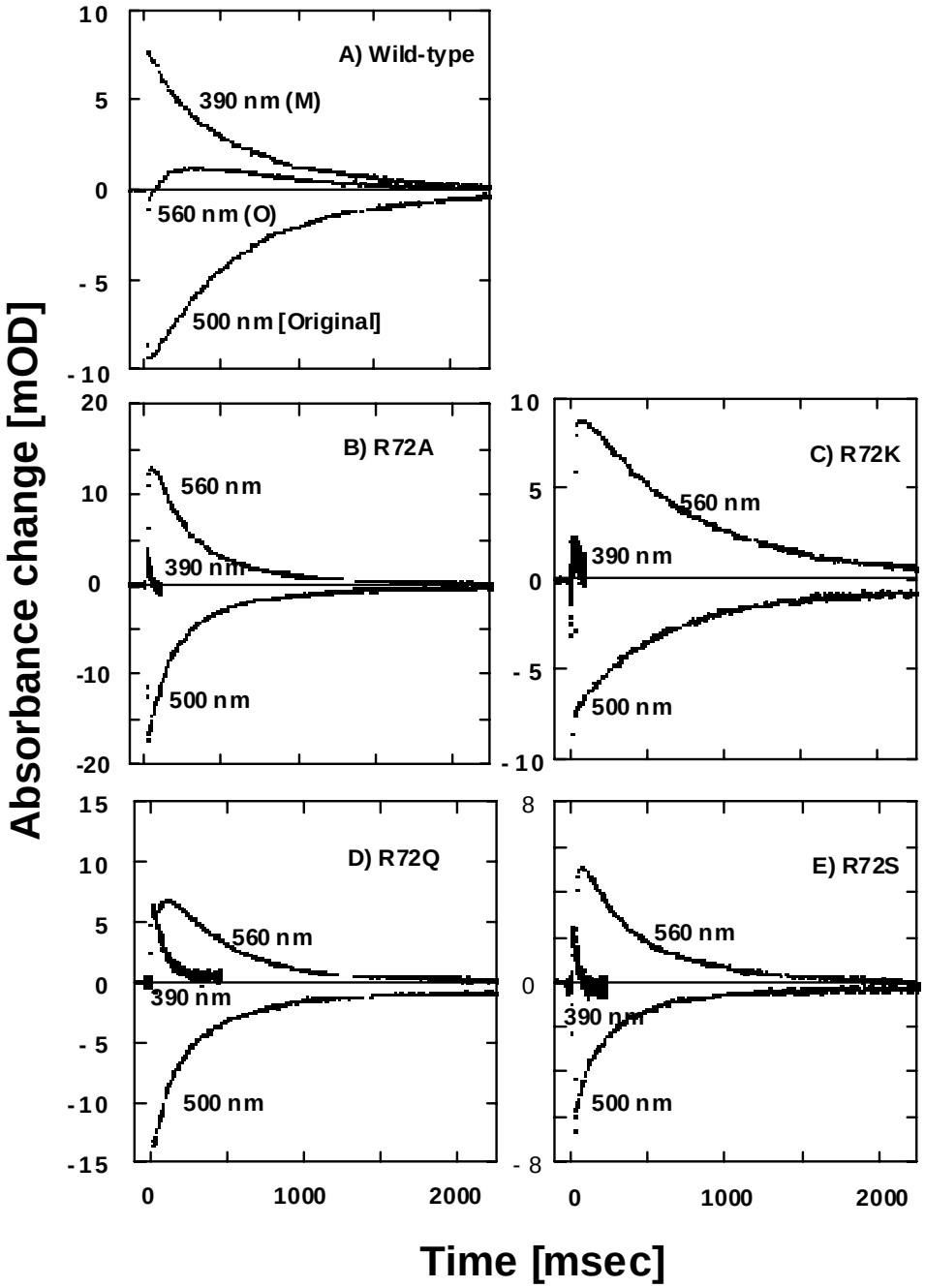


Figure 4 (Ikeura et al.)

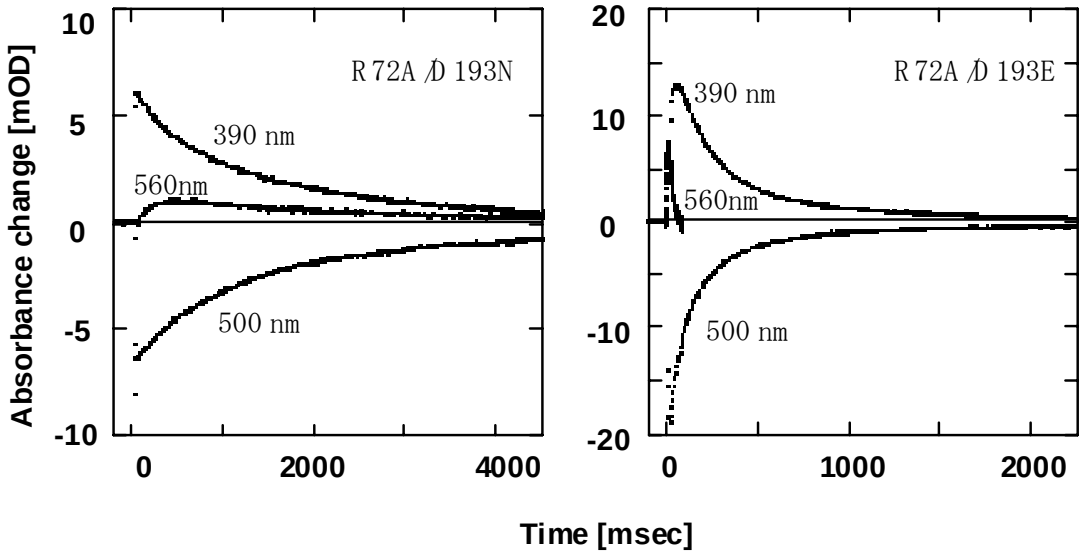


Figure 5 (Ikeura et al.)

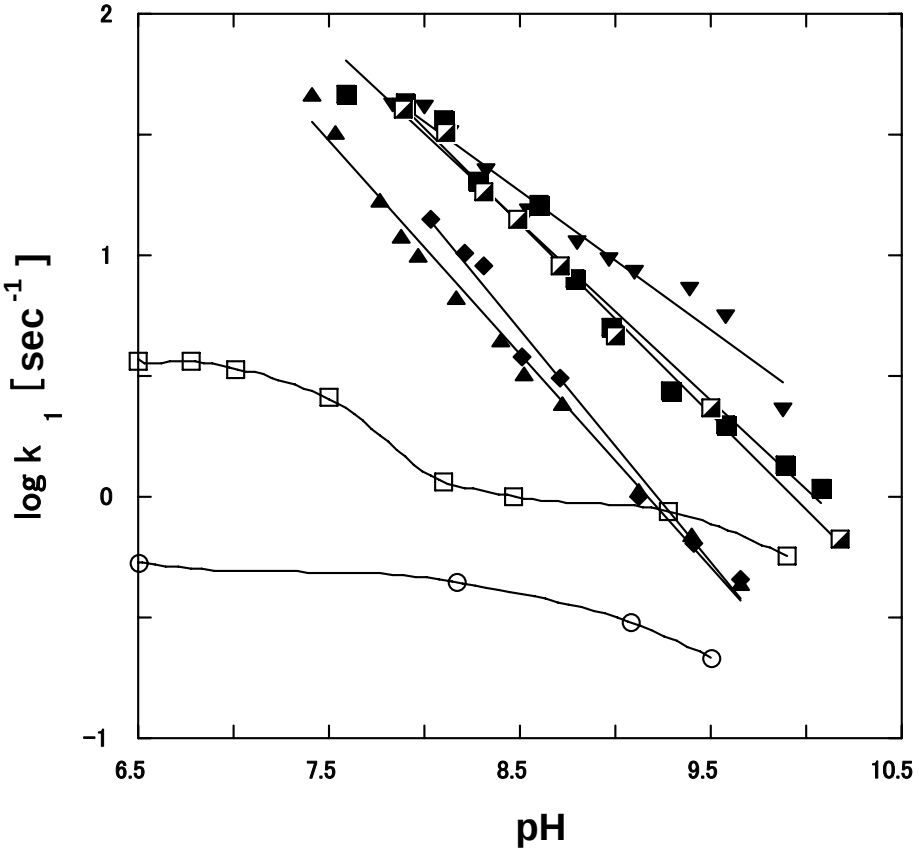


Figure 6. (Ikeura et al.)

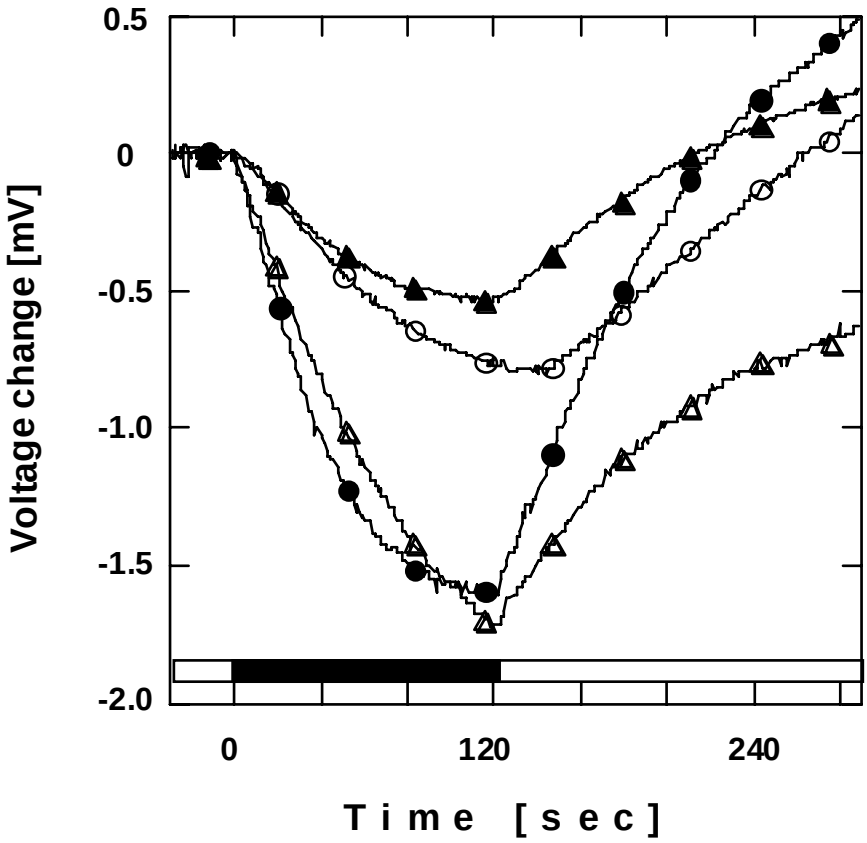


Figure 7. (Ikeura et al.)