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Influence of Arg72 of *pharaonis* Phoborhodopsin on M-intermediate Decay and Proton Pumping Activity

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X-ray structures of *pharaonis* phoborhodopsin (*p*pR) show the different direction of the side chain of Arg72 from that of the corresponding residue (Arg82) of bacteriorhodopsin, BR. For BR, this residue is considered to play an important role in the proton pumping. In order to investigate the role of Arg72 in *p*pR, we constructed Arg72 mutants of R72A, R72K and R72Q, and measured the photocycle and proton pumping activities. The pH-titration curves on the absorption maximum of the mutants were shifted to alkaline in comparison of that of the wild-type. This may imply the increase of pKa of D75, suggesting the presence of the (probably electric) interaction between D75 and Arg72. Rate constants of the M-decay were 3-7 times faster than that of the wild-type, and the time for the completion of the photocycling was also reduced. Using SnO_2 electrode, the rate of transmembrane proton transport was measured upon illumination. The photo-induced proton pumping activities were estimated after the corrections that are the percentages of the associated form of D75 (which has no pumping activity) and the photocycling rates. R72A and R72Q showed the reduced activity while R72K did not reduce the activity.

Key words: Super-conserved Arg, Photocycling, pKa of the counterion of the Schiff base, Hydrogen bonding network, SnO_2 electrode, Archaeal rhodopsin, Sensory rhodopsin.

INTRODUCTION

pharaonis phoborhodopsin (*p*pR or *pharaonis* sensory rhodopsin II, psRII) is a photophobic receptor of *Natronobacterium pharaonis* [1] and is a protein corresponding to phoborhodopsin (pR) in *Halobacterium salinarum*[2]. *p*pR is stable in comparison with pR, especially in dilute salt solutions. It is noteworthy to describe that the functional expression in *Escherichia coli* membrane has been succeeded [3].

X-ray structure of *p*pR was solved, which showed the different direction of the side chain of $Arg72^{ppR}$ of *p*pR from that of the corresponding residue of BR [4,5]. This Arg residue is super-conserved among all archaeal retinal proteins [6]. In BR, the $Arg82^{BR}$ side chain faces negatively charged $Asp85^{BR}$ in the ground state, and turns its direction oppositely toward the extracellular surface at the M-intermediate [7]. This flip-flop of this residue is considered important for the proton transport. As described above, the side chain of $Arg72^{ppR}$ faces the extracellular surface even in the ground state [4,5]. This different direction of $Arg72^{ppR}$ is proposed to be the origin of the blue-shifted absorption maximum [4].

In this article, in order to examine the role of this Arg residue in *p*pR, we prepared mutants of R72A, R72Q and R72K, and measured the photocycling rate and the transmembrane proton transport activity. The observations obtained are interpreted as follows: we assume the hypothesis proposed previously that a proton comes through both the cytoplasmic (CP) and extracellular channel (EC) to the deprotonated Schiff base at the M-decay [8]. Under this assumption, we concluded that the ratio of the proton movement through EC to that through CP increases due to the lack of the positive charge of Arg or Lys in EC for the Arg-mutants except for R72K. Since CP is very hydrophobic, the proton transfer rate of EC may be much larger than in CP. Hence, the M-decay rate becomes faster for R72A and R72Q.

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MATERIALS AND METHODS

Sample preparations. The expression of histidine-tagged recombinant *pp*R in *E. coli* BL21(DE3) and its purification were described previously [9]. To prepare mutant genes Quick change™ Site-Directed Mutagenis Kit (STARATAGENE) was used and a template was pET/*pp*RHis. For measurements of the M-decay rate, the protein was reconstituted into phosphatidyl choline liposomes (molar ratio of 1:50). *Inside-out membrane vesicles derived from E. coli* cells were prepared with a French press as described [8].

Measurements. An apparatus and methods for the flash-photolysis were the same as described [10]. The light-driven proton transport across the membrane vesicles was measured using SnO_2 electrode which is very sensitive to the pH change. The sample was suspended in 400 mM (pH 7) containing no buffer. The actinic light (> 520 nm) was provided from 300-W xenon arc lamp through a combinations of filters (HA50, IRA05, Y52 and CM500). The intensity was 0.4 kW/m^2. The electrical potential difference was measured with a potentiostat/galvanostat model 2000 (Toho Technical Res., Tokyo) operated in a potential measurement mode. A 1-Hz low-cut electric filter was used.

RESUTLS AND DISCUSSION

The absorption spectra in the ground state were measured at various pH from 12 to 2. For determining the fraction of orange form, the increase in absorbance at 541 nm was plotted as the fraction of its maximum value for the preparation as a function of pH. The curves of the fraction of red form against pH were shifted to alkaline in comparison with that of the wild-type, meaning that the Arg residue controls pKa of D75 whose deprotonated state works as a counterion of the positively charged protonated Schiff base. Since, according to the X-ray structure, no hydrogen-bonding network seems to exist between R72 and D75, this pKa change may be due to the electrical interaction between them. The positive charge of guanidinium decreases pKa of D75. The curve of R72K was complicated due maybe to the protonation of the Lys residue itself.

Figure 1 shows the flash-photolysis data on the M-decay of the wild-type, R72A and R72Q. The decay time constants were 9, 59 and 26 s^{-1} for the wild-type, R72A and R72Q, respectively, revealing the fast M-decay of the mutants. The values are those of *pp*R reconstituted into liposomes, because the proton-pumping activity described below was measured in membrane vesicles and since the M-decay rates of vesicles were not measurable due to their strong scatter of the flash-light. For solubilized samples with dodecyl-β-D-maltoside, the similar faster M-decays were obtained than that of the wild-type. For R72K, flash-induced amounts of the M were too small to estimate the decay constant. The reason is not clear, but the most probable reason is the presence of a very stable photo-intermediate prior to M. Detailed analysis is awaited in future.

The rise and decay of M-intermediate is corresponding to the deprotonation and reprotonation of the Schiff base, respectively. Upon the formation of M of BR, a proton is transferred from the Schiff base to Asp85^{BR} with the simultaneous proton release from the so-called proton releasing group located at the extracellular surface. In the next step, Asp96^{BR} donates its proton to the deprotonated Schiff base. Asp96^{BR}, then, receives proton from the cytoplasm to form O-intermediate. At the O-decay, the proton-transfer from Asp85^{BR} to the proton releasing group occurs. Thus, after the completion of one cycle, proton is transported effectively from cytoplasm to extracellular space. This prompt transfer of a proton is supported by the hydrogen-bonding network. The Arg82^{BR} side chain participates in this network [7].

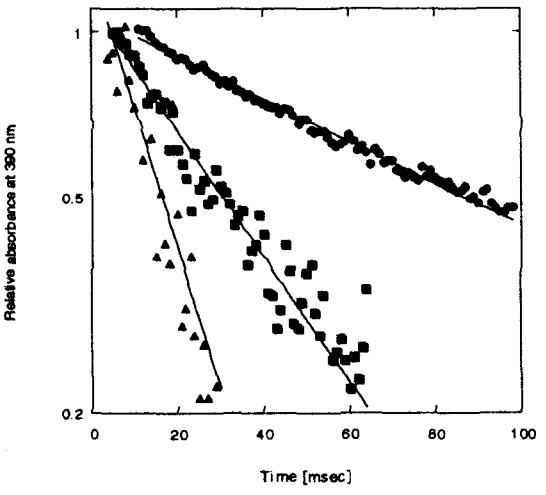

Figure 1. M-decay kinetics monitored at 390 nm. Purified proteins of ppR were reconstituted in phosphatidyl choline liposomes which were suspended in 400 mM NaCl (pH 7.0). Circle, wild-type; triangle, R72A; square, R72Q.

In *pp*R, the hydrogen-bonding network is disrupted, one of whose reasons is the orientation of the guanidinium of Arg72^{ppR}. The proton transfer mechanism in BR may suggest that this Arg72^{ppR} affects the photochemistry prior to M and/or the proton-releasing event [11]. Actually, the proton-release was proven to occur at O-decay of *pp*R [12], which is consistent with the lack of the network. However, although it is so simple, Arg72^{ppR} in EC might not affect the M-decay because a proton comes to the

Schiff base from CP in BR, which is contradictory to the present observation.

The reason for the slow M-decay of *pp*R is the lack of the proton-donating Asp corresponding to $Asp96^{BR}$ and hence high hydrophobicity of CP [12]. Proton-transfer rate in CP might be slower. However, since the weak proton-pumping activity of the wild-type was observable [8], we concluded that, at M-decay, protons come to the deprotonated Schiff base though both CP and EC. The proton-pumping process is originated from the proton transfer through CP. If we accept this idea, the present observation may be interpreted as follows: The mutation of $Arg72^{ppR}$ located in EC increased the proton current through EC, which leads to the faster M-decay. This hypothesis forecasts the decrease in the photo-induced proton-pumping.

Table 1. Data for the calculation of the photo-induced proton-pumping activity of Arg72 mutants

	wild-type	R72A	R72K	R72Q
$\tau_{1/2}$ of photocycle[ms]	530	210	230	180
Fraction of orange form [%]	95	55	70	55
Initial rate of proton pump [mV / sec / mg protein]	0.26	0.24	0.50	0.22
Normalized proton pump activity	1	0.65	1.12	0.47

The values listed were measured in inside-out membrane vesicle expressing *pp*R suspending in 400 mM NaCl (pH 7.0) excepted for fraction of orange form. The value of fraction of orange form was measured in PC liposome.

Therefore, we undertook to estimate the photo-induced proton-pumping activity. The values required for the estimation are listed in Table 1. The activity depends on the turn-over numbers of the photocycling, and this was assumed to be reciprocally proportional to the half-time of the cycling. The bathchromic species in which D75 is protonated does not possess the activity, and, then, the fraction of an orange form (where D75 is deprotonated) was estimated from the pH titration curve as described above. The rate in the SnO_2 electrode potential upon illumination was used as a measure of the proton transport rate through membrane vesicles. The proton-pump activity was calculated as (initial rate of proton transport) x (the half-time) / (fraction of the orange form), and normalized activities are listed in the lowest line in the table. This table reveals clearly the decrease in the proton-pumping activity of R72A and R72Q. On the other hand, it is of interest that the activity of R72K did not decrease. For R72K and the wild-type, there exists the positively charged residue (Arg or Lys) in EC, which might hinder the proton transfer to the deprotonated Schiff base through EC. In addition, the decay of O-intermediate seemed not to be affected by the Arg-mutation. The reason for the unchanged O-decay rate in spite of the 7-fold change in the M-decay of R72A mutant is not clear at present and is an interesting further study.

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