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Positioning proton-donating residues to the Schiff-base accelerates the M-decay of *pharaonis* phoborhodopsin expressed in *Escherichia coli*

Masayuki Iwamoto, Kazumi Shimono, Masato Sumi, Naoki Kamo*

Laboratory of Biophysical Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

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Abstract

Phoborhodopsin (also called sensory rhodopsin II, sR-II) is a receptor for the negative phototaxis of *Halobacterium salinarum* (pR), and *pharaonis* phoborhodopsin (*p*pR) is the corresponding receptor of *Natronobacterium pharaonis*. pR and *p*pR are retinoid proteins and have a photocycle similar to that of bacteriorhodopsin (bR). A major difference between the photocycle of the ion pump bR and the sensor pR or *p*pR is found in their turnover rates which are much faster for bR. A reason for this difference might be found in the lack of a proton-donating residue to the Schiff base which is formed between the lysine of the opsin and retinal. To reconstruct a bR-like photochemical behavior, we expressed *p*pR mutants in *Escherichia coli* in which proton-donating groups have been reintroduced into the cytoplasmic proton channel. In measurement of the photocycle it could be shown that the F86D mutant of *p*pR (Phe86 was substituted by Asp) showed a faster decay of M-intermediate than the wild-type, which was even accelerated in the F86D/L40T double mutant.

Keywords: *Pharaonis* phoborhodopsin (*p*pR); *Pharaonis* sensory rhodopsin (*p*sR-II); Photocycle; M-decay of mutants; Expression in *E. coli*

1. Introduction

Halobacterium salinarum possesses four retinal proteins. The two ion-pumps, bacteriorhodopsin (bR) [1,2] and halorhodopsin (hR) [3,4] as well as the two photosensors, sensory rhodopsin (sR or sR-I) [5–7] and phoborhodopsin (pR, also called sR-II) [8–12]. Although the first three retinal proteins have been studied in great detail, much less is known about pR, the negative phototaxis receptor.

We [13–16] and Engelhard et al. [17–20] have succeeded in the purification of a pR-like protein

* Corresponding author. Tel.: +81-11-726-3923; fax: +81-11-706-4984.
E-mail address: nkamo@pharm.hokudai.ac.jp (N. Kamo)

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from *Natronobacterium pharaonis* and have characterized the protein in great detail. It was termed *pharaonis* phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, *p*sR-II). Recently, we [21] succeeded in the functional expression of *p*pR in *Escherichia coli*. This provides large amounts of the protein itself and its mutants and permits the investigation of the photochemistry of *p*pR at a molecular level [22].

On light excitation, the bacterial rhodopsins react in a cyclic manner, forming a series of intermediates before returning back to the original state. The photochemistry of *p*pR (or pR) includes, similar to bR, K-, L-, M- and O-like intermediates [20,23]. However, the photocycling rate of pR or *p*pR is almost two orders of magnitude slower than that of bR [20,23]. Comparing the primary sequence of bR and *p*pR it becomes evident that a change of an amino acid(s) in the cytoplasmic channel might be responsible for these differences. Asn96 and Thr46 are crucial for the proton-pumping mechanism of bR. In *p*pR these amino acids are replaced by Phe (F86) and Leu (L40), respectively [18]. In the M-state, the Schiff base is deprotonated and it is thought that the decay of M coincides with the reprotonation of the Schiff base by the hydrogen-bonding chain which includes Asp96 and Thr46 [24]. The absence of these internal proton donors in *p*pR may account for the slow decay of M. Hence replacement of F86 by Asp and L40 by Thr might restore a bR-like photocycle. The present communication reports that site-directed mutagenesis of *p*pR to acquire the internal proton donor and possible hydrogen-bonding network accelerated the M-decay as much as approximately 36-fold at pH 5.0 and 130-fold at pH 7.0.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli JM109 was used as a host for DNA manipulation, and BL21(DE3) was used for gene expression. Cells were grown in $2\times$ YT medium supplemented by ampicillin (final concentration of 50 μg/ml).

2.2. Preparation of F86D mutant

The gene for the single amino acid replacement was prepared by PCR. For PCR, two pairs of sense and antisense oligonucleotide primers were designed based on the nucleotide sequence in the GenBank™ data base (accession no. z35086); psp (5′-*CATATG*GTGGGACTTAC-GACC-3′) and pap86d (5′-CAGC*CCTAGG*TCGTAGACGATG-3′) and psp86d (5′-CGA*CCTAGG*GCTGCTTGCGGGG-3′) and pap, (5′-AGAATAACGACGGGACGT-TCG-3′). Underlined bases indicate the added restriction sites *Nde*I and *Bln*I. The plasmid containing a full-length of *psopII* (opsin of *pharaonis* sensory rhodopsin II, *pharaonis* phoborhodopsin) was used as a template for PCR. Two PCRs were performed. One used psp and pap86d as primers, and the other psp86d and pap. Two PCR products were obtained, purified and subcloned into the plasmid vector pGEM-T Easy (Promega). The *Bln*I and *Nco*I fragments from the latter plasmid were ligated to the *Bln*I and *Nco*I sites of the former plasmid. The *Nde*I and *Not*I fragments from this plasmid were ligated to *Nde*I and *Nco*I sites of pET21 (Novagen).

2.3. Preparation of L40T and F86D / L40T mutants

The Kunkel method [25] was used to prepare L40T and F86D/L40T mutants. As templates, plasmid vector pGEM-T Easy containing the full-length of *psopII* or F86D gene (as described before) was used. For the Kunkel method, an antisense, pap40t (5′-CTGATGCCGA-CAGTCGT*TACGTA*GTACCGT-3′) oligonucleotide primer was designed based on the nucleotide sequence in the GenBank™ data base. Underlined is the added restriction site *Sna*BI.

2.4. DNA sequencing

DNA sequencing was carried out using a kit (Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) to confirm the correct mutation. The PCR products were analyzed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

2.5. Expression of the wild-type and mutant protein

The wild-type and mutant *p*pR were expressed in *E. coli* BL21(DE3) by induction of the addition of 1 mM IPTG and 10 μM all-*trans* retinal. The preparation of membranes has been described previously [21].

2.6. Flash spectroscopy

Sample preparations, the apparatus and the procedure were essentially the same as described earlier [21]. Buffer solutions were: for pH 5.0, 20 mM citrate–HCl, 4.0 M NaCl and 0.5% octylglucoside; for pH 7.0, 50 mM potassium-phosphate buffer, 4.0 M NaCl and 0.5% octylglucoside; for pH 9.0, 50 mM CHES–NaOH, 4.0 M NaCl and 0.5% octylglucoside. Absorbance changes at 390, 500 and 560 nm mainly monitor $p\mathrm{pR_M}$, *p*pR and $p\mathrm{pR_O}$, respectively, but the contributions from other components cannot be ignored. Time constants were calculated by taking the contribution from other components into accounts as described elsewhere [13].

3. Results and discussion

Fig. 1A shows typical flash-photolysis data of the F86D mutant (pH 5.0) and the wild-type (Fig. 1B) at selective wavelengths. The absorption spectra of the mutants F86D, L40T and F86D/L40T are indistinguishable from that of wild-type *p*pR (data not shown). The small value of the ordinate of Fig. 1A resulted from the small amount of the mutant, and the reason for it not being expressed at high levels is not known at present. Time constants calculated in accordance with a photocycling scheme proposed previously [13] are listed in Table 1. Comparison with data of the wild-type (Fig. 1B) under the same conditions reveals that substitution accelerates the decay of $p\mathrm{pR_M}$ (M-decay). The k_1 values of the F86D mutant are generally larger at any pH than those of the wild-type, although the difference of alkaline pH is negligible. The table also reveals a slower decay of $p\mathrm{pR_O}$ (k_2) in the mutant than in the wild-type, but the reason for this is not yet known.

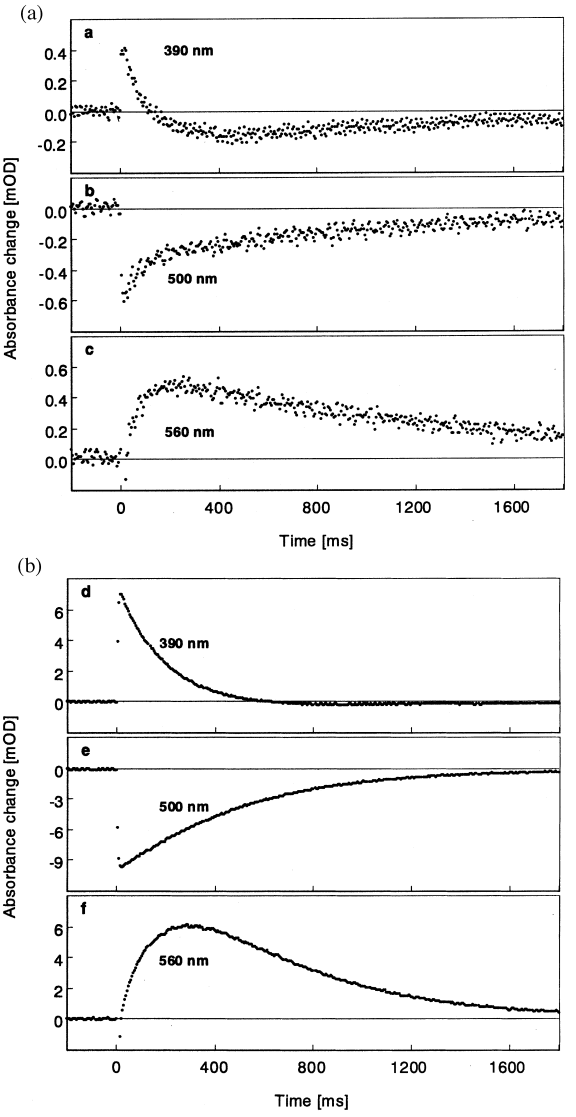

Fig. 1. Typical flash-photolysis data of the F86D mutant (A) and the wild-type *p*pR (B) at selective wavelengths. $p\mathrm{pR_M}$ was monitored at 390 nm (a and d). The recovery of the original state *p*pR was observed at 500 nm (b and e) and the O-intermediate $p\mathrm{pR_O}$ was monitored at 560 nm (c and f). The ordinate values depict the change in the absorbance caused by the flash excitation. Measurements were done at 20°C and pH 5.0; Further details see Section 2.

In bR it has been shown that a substitution of Asp96 by Asn slows the M-decay rate consider-

Table 1
Time constants of the decay of ppR$_M$ and ppR^a_O

	pH 5.0		pH 7.0		pH 9.0	
	k_1	k_2	k_1	k_2	k_1	k_2
Wild-type	5.2	2.2	0.75	1.3	0.46	1.2
F86D	11	0.83	2.3	0.91	0.69	0.78
F86D/L40T	190	0.66	97	0.86	13	2.7

[a] k_1 and k_2 stand for the time constant of the decay of ppR$_M$ and ppR$_O$, respectively, with the unit of s^{-1}. Buffer compositions are as in Section 2. Temperature was 20°C.

ably [26,27]. This has been interpreted as follows: The protonated carboxyl group of Asp96 functions as a proton-donor to the deprotonated Schiff base of the M-intermediate. Therefore, reprotonation of D96 occurs at the N → O step. The lack of a carboxyl group like in the D96N mutant slows the decay rate [28,29]. Since Phe86 of ppR occupies the position of Asp96 in bR, the introduction of a carboxyl group like in the F86D mutant should accelerate the reprotonation of the Schiff base. Thus, the result in Fig. 1 and Table 1 agree with the notion that the slow decay of ppR$_M$ is caused by the lack of a residue which donates a proton to the deprotonated Schiff base. The substitution increased the ppR$_M$-decay approximately three − or twofold under a neutral and acidic condition, but was small under an alkaline condition. Engelhard et al. [30] presented preliminary results on the M-decay of the F86D mutant expressed in *Halobacterium salinarum*, and reported no appreciable change in the decay. This discrepancy from our results merits further investigation; possible reasons are the difference in membrane lipids or the duration of the flash.

The distance between Asp96 and Schiff base in bR is 1.2 nm [31], too far for a direct H^+ transfer to the Schiff base. Hydrogen-bonded networks between Asp96 and the Schiff base has been proofed to be important for fast proton transfer [32]. The current bR structure places Thr46 in helix B and Asp96 in helix C at similar distance within the membrane, facing each other at a distance of approximately 0.33 nm [31]. In the bR-mutant T46V, M-decay is accelerated whereas the N-decay is slowed [33,34]. This result has been interpreted in terms of the influence of Thr46 on the deprotonation and reprotonation of Asp96. Thus, Thr46 is assumed to interact with Asp96 which is connected to the Schiff base via several intervening water molecules [35,36]. This hydrogen bond accounts for the unusually high pK_a of Asp96 [33], and the Thr46–Asp96 domain together with the intervening water molecules determines the rate of the M-decay [36].

The corresponding residue to Thr46 of bR is Leu40 in ppR [18]. Thus, we substituted Leu40 with a Thr. Fig. 2 shows the results of the M-decay measurement of F86D/L40T double mutant, as well as those of the wild-type and F86D mutant for comparison. The figure shows that the M-decay of the double mutant is much faster than that of F86D single mutant. The decay constants are listed in Table 1; the M-decay constant was 190 s^{-1} ($T_{1/2} = 3.6$ ms) at pH 5, close to that of the wild-type bR (300–200 s^{-1}). This finding indicates that like in bR, the proton translocation from the proton donor (Asp86 of F86D mutant) to the Schiff base is mediated by Thr40 (possibly as well as some water molecules) in the F86D/L40T mutant. It should be noted that the time constants of the O-decay are not affected by the mutation from F86D to F86D/L40T.

The M-decay of T46V of bR is faster than that of the wild-type; replacement of Thr with Val accelerates the decay [33,34]. In ppR, however, the M-decay of the L40T mutant was faster than that of the wild-type in acidic media and did not change in alkaline media: the time constants were 35, 2.8 and 0.40 s^{-1} at pH 5.0, 7.0 and 9.0, respectively. In this case, the role of Thr seems to be opposite, suggesting different coordination of water molecules in the F86D–L40T domain of the ppR mutant compared to the Asp96–Thr46 domain of bR.

In the present communication, we showed that the substitution of F86 and L40 residues of ppR those found on bR causes an acceleration in the M-decay rate, and that the rate in the double mutant is close to that of the wild-type bR. From the stand point of the M-decay rate, in the double mutant the hydrogen-bonded chain for intra-

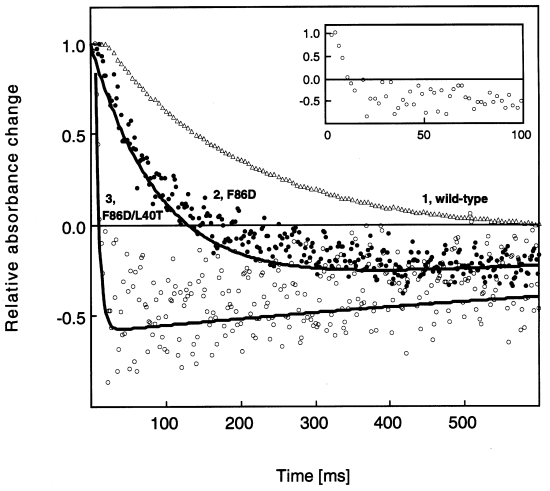

Fig. 2. M-decay kinetics monitored at 390 nm for the wild-type (1), F86D (2) and F86D/L40T (3). Inset shows the kinetic data of F86D/L40T in the earlier time range. The maximum amplitude of each curve (amplitude immediately after the flash) was adjusted to unity. The values of k_1 (decay of $p\mathrm{pR_M}$) are listed in Table 1. The dots are experimental data points obtained whereas the thick lines in (2) and (3) represent the simulate curves using the k_1 and k_2 values listed in Table 1. The low signal to noise ratio of (2) and (3) are due to the small expression yields of these mutants.

molecular proton transport from the cytoplasmic side to the deprotonated Schiff base might be realized. The deprotonation of the Schiff-base and the simultaneous protonation of Asp75 (corresponding to Asp85 of bR) has been shown [19]: Assuming that proton release occurs from the extracellular part of the protein, the vectorial proton transport from the cytoplasm to extracellular side of the protein will occur. Furthermore, sensory rhodopsin I pumps protons when it is free from Htr-I (halobacterial transducer-I) [37]. The data presented in this paper and the observation that sR-I can function as a poor proton pump indicate that only a few residues are responsible for efficient proton transfer. It is interesting to elucidate if the *p*pR-mutants described in this paper are able to pump protons, thereby creating a membrane potential. It is noteworthy that Engelhard and Bamberg found a photo-induced membrane potential for the F86D mutant (personal communication).

4. Nomenclature

br:	bacteriorhodopsin
CHES:	2-cyclohexil-amino-ethanesulfonic acid
F86D mutant:	mutant in which Phe86 is substituted by Asp
IPTG:	isopropyl-1-thio-β-galactoside
*p*pR:	*pharaonis* phoborhodopsin
$p\mathrm{pR_M}$:	M-intermediate of *p*pR
$p\mathrm{pR_O}$:	O-intermediate of *p*pR
sR:	sensory rhodopsin

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