Postprint: Effects of M145F/F146M Mutation on the Photocycles of the Photoreceptor Proteins Bacteriorhodopsin and Archaerhodopsin-4

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Abstract

Archaerhodopsin 4 (aR4) and bacteriorhodopsin (bR) both belong to the family Halobacteriaceae, share 59% sequence homology, and function as light-driven proton pumps. Their physiological role is to translocate protons from the intracellular to the extracellular space under illumination, thereby establishing a transmembrane proton gradient that is harnessed by the membrane-bound ATP synthase for ATP synthesis, thus accomplishing the conversion of light energy into biological energy. Although aR4 and bR exhibit similar photocycle mechanisms, their proton transfer sequences differ: aR4 first captures a proton from the intracellular side before releasing it extracellularly, whereas bR follows the reverse order. Methionine-145 constitutes a critical residue within the retinal chromophore binding pocket of bR that substantially influences its photocycle, while Phenylalanine-146 at the corresponding position in aR4 represents the sole residue in the retinal binding region that differs from bR. Consequently, site-directed mutagenesis combined with comparative analyses employing UV-Vis absorption spectroscopy, kinetic spectroscopy, proton pump functional assays, and low-temperature transmission infrared spectroscopy to examine the impact of M145F and F146M single-point mutations on the photocycles of bR and aR4 provides valuable insights into the structure-function relationships of aR4. The findings reveal that the M145F mutation leads to the loss of the L intermediate in the bR photocycle and attenuates proton pump activity, whereas the F146M mutation exerts no significant effect on the aR4 photocycle, and the proton release sequence in aR4 remains unaltered post-mutation, suggesting that the functional role of this residue is not conserved between the two systems.

Full Text

Effect of M145F/F146M Mutations on the Photocycle of Photoreceptor Proteins Bacteriorhodopsin and Archaerhodopsin 4

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Abstract

Both archaerhodopsin 4 (aR4) and bacteriorhodopsin (bR) belong to the Halobacteriaceae family, sharing 59% homology, and function as light-driven proton pumps. Their function is to pump protons from the intracellular side to the extracellular side under illumination, forming a transmembrane proton gradient that is utilized by ATP synthase for ATP synthesis, thereby completing the conversion of light energy to biological energy. aR4 and bR have similar photocycles, but differ in their proton transfer timing: aR4 first absorbs a proton from the cytoplasm and then releases it to the extracellular side, whereas bR does the opposite. Methionine-145 is an important residue located in the retinal chromophore binding region of bR and significantly influences its photocycle. Phenylalanine-146 is the corresponding residue in aR4 at the same position and is the only residue in the retinal binding region that differs from bR. Therefore, through site-directed mutagenesis and comparative analysis using UV-Vis absorption spectroscopy, kinetic spectroscopy, proton pump function detection, and low-temperature transmission infrared spectroscopy, this study investigates the effects of M145F and F146M single-point mutations on the photocycles of bR and aR4 to deepen understanding of the structure-function relationship in aR4. The results show that the M145F mutation causes loss of the L intermediate and weakened proton pump function in bR, while the F146M mutation does not significantly affect the aR4 photocycle, and the proton release timing in the mutant does not reverse. This indicates that the residues at this position play different roles in the two systems.

Keywords: Archaerhodopsin 4; M145F/F146M single-point mutation; Photocycle intermediate states; Proton pump; Low-temperature transmission infrared spectroscopy

Introduction

Bacteriorhodopsin (bR) is a retinal protein discovered in *Halobacterium salinarum* of the Halobacteriaceae family and is the earliest discovered and most thoroughly studied light-driven proton pump protein. After photoexcitation, it produces a series of intermediate states and completes a photocycle from the ground state through K, L, M, N, and O states back to the ground state [**?**]. Photoexcitation results in rapid release of a proton to the extracellular side,

followed by proton uptake from the cytoplasmic side [**?**]. bR shares similar seven-transmembrane structures and functional modes with G-protein coupled receptors (GPCRs), and because its structure and function have been well characterized, it is often used as a natural model for studying GPCRs [**?**].

Archaerhodopsin 4 is a bR-like protein extracted from the *Halobacterium* species xz515 strain discovered in Tibetan salt lakes in China, sharing 59% homology with bR [**?**]. Upon photoexcitation, aR4 enters a photocycle similar to bR, producing a series of intermediates. However, the proton uptake and release process during its photocycle is opposite to that of bR. The structure of aR4 has not yet been resolved, but because it shares a trimeric structure with bR [**?**], comparative studies with bR help deepen understanding of its structure-function relationship.

Since the intensity and shape of amide bands are highly sensitive to protein secondary structure and hydrogen bonding networks, Fourier transform infrared spectroscopy (FTIR) has been widely used to study bR structure, photocycle intermediates, and conformational dynamics. Braiman et al. constructed D85E and D96E mutants and compared their infrared difference spectra with wild-type intermediates, first confirming that the appearance of a C=O stretching vibration at 1761 cm^{-1} (+) marked the protonation of D85 in the M state, thereby identifying D85 as the proton acceptor of the Schiff base, while also assigning the 1742/1748 cm^{-1} (-/+) peak to protonated D96 [**?**]. FTIR studies of D96E and D96A mutants revealed that the N-state peak at 1742 cm^{-1} shifted and intensified under alkaline conditions, confirming that D96 is deprotonated in the N state, i.e., D96 serves as the proton donor to the Schiff base [**?**]. Studies on the Y57D mutant using infrared and Raman spectroscopy suggested that Y57D does not involve Schiff base deprotonation, and proton transfer is achieved through interaction with D212 [**?**].

Methionine-145 (M145) is an important residue located in the retinal chromophore binding region of bR and significantly influences its photocycle. Compared with wild-type bR, the M145F mutation changes the all-trans to 13-cis ratio of retinal in the dark-adapted state [**?**] and affects L-state formation [**?**]. The possible reason is that the M145 mutation affects the interaction between W182 and the methyl group on the retinal side chain, thereby influencing the conversion of retinal from all-trans to 13-cis [**?**]. Phenylalanine-146 (F146) is the residue in aR4 corresponding to M145 in the retinal chromophore binding region and is the only residue in this region that differs from bR. The effect of F146 on the aR4 photocycle and whether it is related to aR4' s proton transfer timing have not been reported. Therefore, this study constructed M145F and F146M mutants and used Fourier transform infrared spectroscopy combined with other spectroscopic techniques to comparatively analyze the roles of M145F and F146M, providing preliminary insights into the role of F146 in aR4 proton transport.

Materials and Methods

1.1.1 Strains and Plasmids

The bR expression-deficient strain *Halobacterium* species L33 and the halophilic archaeon *Halobacterium* species xz515 expressing aR4 used in this study were kindly provided by Professor Jian-Dong Ding of Fudan University.

1.1.2 Enzymes and Reagents

T4 DNA ligase, Taq DNA polymerase, Pfu DNA polymerase, DNase I, RNase, restriction endonucleases BamHI and HindIII, DNA Marker, and Protein Marker were purchased from Takara; tetracycline from Shanghai Bioengineering Technology Service; PEG-600 and novobiocin from Sigma; plasmid extraction and gel recovery kits from AxyGen.

1.1.3 Culture Media

1) Peptone liquid medium (PM) per liter: NaCl 250 g, $MgSO_4 \cdot 7H_2O$ 20 g, sodium citrate 3 g, KCl 2 g, $CaCl_2$ 0.2 g, Oxoid L37 10 g, pH 7.4
2) Regeneration liquid medium per liter: PM medium supplemented with 15% sucrose and 50 mM Tris-HCl, pH 7.2
3) Regeneration semi-solid medium: regeneration liquid medium with 0.6% agar
4) Regeneration solid medium: regeneration liquid medium with 1.2% agar
5) Protoplast formation solution: 15% sucrose, 2 M NaCl, 27 mM KCl, 50 mM Tris-HCl, pH 8.8

1.1.4 Buffer

2 mM phosphate buffered saline (PBS) per liter: NaCl 1.6 g, KCl 0.04 g, Na_2HPO_4 0.288 g, KH_2PO_4 0.048 g, 0.025% NaN_3, pH 7

1.2.1 PCR Primer Design

Site-directed mutagenesis of bop and ar4 genes was performed by PCR using the following primers:

Mutant construction was performed as described in reference [**?**]. The recombinant expression vectors were named pNor-M145F-bop and pNor-F146M-ar4.

1.2.3 Halophile Homologous Transformation and Screening

Halophile transformation was performed as described in reference [**?**].

1.2.4 Protein Expression and Purification

Proteins were cultured in PM liquid medium and purified using the method described in reference [**?**]. The obtained proteins were named RCL33-aR4,

F146ML33-aR4, RCL33-bR, and M145FL33-bR (RC, Recombinant). After purification, molecular weight was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was measured by the Bradford method.

1.2.5 UV-Vis Spectroscopy

Appropriate protein samples were suspended in buffer containing 100 mM NaCl and 20 mM KCl at pH 7, and UV-Vis absorption spectra of purified proteins were measured using a UV-Vis spectrophotometer (T6 New Century).

1.2.6 Flash Kinetic Spectroscopy

Protein solutions of appropriate concentration were prepared in buffer containing 100 mM NaCl and 20 mM KCl at pH 7. Light-induced transient absorption changes were measured at room temperature using a homemade kinetic spectrometer (also called flash spectrometer). A camera flash served as the excitation source, and measuring light was provided by a halogen lamp perpendicular to the excitation light. The M state was monitored at 412 nm, the O state at 660 nm, and all samples were converted to the light-adapted state. Multiple acquisitions and averaging were performed to improve signal-to-noise ratio.

1.2.7 Proton Pump Function Detection

Proton pump function was detected using pyranine, a pH-sensitive dye that responds to minute pH changes. By comparing absorbance changes at 456 nm of protein suspensions with and without the dye, the sequence of proton release and uptake during the photocycle was determined.

1.2.8 Low-Temperature Transmission Infrared Spectroscopy

Protein solutions (0.5 mM) were prepared in 2 mM PBS buffer at pH 7. A 100 μL sample was dropped onto a CaF_2 window and dried to form a 10 mm spot. After rehydration with distilled water, the window was mounted in a Specac K22786 low-temperature transmission cell for temperature control. The K, L, M, and N intermediate states were controlled at 90 K, 170 K, 210 K, and 250 K, respectively. Spectra were collected using a Nicolet 8700 FTIR spectrometer with 4 cm^{-1} resolution and 1024 scans over the 4000-1000 cm^{-1} range, and processed using OMNIC 8.0 software.

Results and Discussion

2.1 Amino Acid Sequence Alignment of aR4 and bR

The amino acid sequence alignment of aR4 and bR is shown in Figure 1 [Figure 1: see original paper]. The two proteins share 59% homology, with residues marked in red and blue representing identical and different residues in the retinal binding pocket, respectively.

2.2 Molecular Weight Determination by SDS-PAGE

The purified proteins were analyzed by SDS-PAGE as shown in Figure 2 [Figure 2: see original paper]. M145FL33-bR and F146ML33-aR4 showed the same molecular weight (26 kDa) as RCL33-bR and RCL33-aR4, indicating successful expression of M145FL33-bR and F146ML33-aR4 in the L33 system.

2.3 UV-Vis Absorption Spectroscopy

The UV-Vis absorption spectra of recombinant bR and aR4 proteins are shown in Figure 3 [Figure 3: see original paper]. The M145FL33-bR mutant exhibited a 4 nm blue shift (560 nm) compared with its recombinant wild-type counterpart RCL33-bR (564 nm), while F146ML33-aR4 (547 nm) showed a 3 nm red shift relative to RCL33-aR4 (544 nm). The blue shift in M145FL33-bR indicates that the change in bond order of the retinal chromophore excited state is greater than that of the ground state, resulting in increased distortion compared with the wild type. Conversely, F146ML33-aR4 showed decreased distortion of the retinal chromophore relative to the wild type. Both RCL33-aR4 and F146ML33-aR4 exhibited an M-like state absorption peak at 412 nm, suggesting that aR4 may possess some basal activity, which could be one reason for its weakened proton pump function and warrants further investigation.

2.4 Kinetic Spectroscopy Detection

All samples were resuspended in buffer containing 100 mM NaCl and 20 mM KCl at pH 7, and light-induced transient absorption changes were measured at room temperature as shown in Figure 4 [Figure 4: see original paper].

Compared with RCL33-aR4, the F146ML33-aR4 mutant showed little change in M and O states, with a slightly shorter overall recovery time to the ground state. In contrast, M145FL33-bR exhibited weakened signals for both M and O states compared with RCL33-bR, along with a slightly accelerated M-state decay. The fitted kinetic parameters are summarized in Table 2 .

Crystal structures reveal that during the K-to-L transition in bR, W182 undergoes significant movement toward M145 [14]. The M145F mutation increases local steric hindrance, interfering with the interaction between W182 and the retinal methyl group and hindering the K-to-M transition, thereby weakening the M and O state signals (Figure 5 [Figure 5: see original paper]). In contrast, the F146ML33-aR4 mutation did not significantly affect its M and O states, possibly because the retinal binding cavity in aR4 is larger than in bR, so the F146ML33-aR4 mutation has minimal impact on the arrangement and dynamic conformation of surrounding residues, consistent with aR4 being a weak proton pump (Wang, Ma et al. 2012).

2.5 Proton Pump Function Detection

The proton pump functions of RCL33-aR4 and RCL33-bR are shown in Figure 6 [Figure 6: see original paper]. Table 3 lists the proton pump recovery times obtained by exponential fitting of the data traces in Figure 6. As shown in Figure 6 and Table 3, RCL33-aR4 and RCL33-bR exhibit opposite proton transfer sequences: RCL33-bR releases protons before uptake, while RCL33-aR4 absorbs protons before release, with significantly weaker signal intensity and shorter overall proton uptake/release time, consistent with their differences in proton transfer capability.

Neither F146ML33-aR4 nor M145FL33-bR showed altered proton transfer sequences compared with their respective recombinant wild-type proteins, but changes in proton pump recovery time were observed. The proton pump recovery time for F146ML33-aR4 was 2.09 ms, slightly prolonged compared with RCL33-aR4, with little change in signal intensity. M145FL33-bR showed a significantly shortened recovery time of 2.17 ms compared with RCL33-bR, with markedly reduced signal intensity. The M145F mutation substantially affects proton transfer rate and the ease of proton uptake/release, whereas the F146M mutation has minimal impact, indicating that M145 plays a more important role in bR proton transfer than F146 does in aR4.

2.5 Low-Temperature Transmission Infrared Spectroscopy

Low-temperature transmission infrared spectroscopy was further employed to comparatively study the photocycle intermediates of recombinant proteins, as shown in Figures 7 [Figure 7: see original paper]-8. Peaks at 1255, 1216, 1202, and 1169 cm^{-1} can be assigned to C-C stretching vibrations of all-trans retinal chromophore [15], while the peak at 1194 cm^{-1} corresponds to mixed stretching vibrations of 13-cis retinal [16]. In the M and N states, deprotonation of the Schiff base shifts this peak to 1186 cm^{-1}. In the K state, the pair at 1514 (+)/1529 (-) cm^{-1} is assigned to C=C stretching vibrations of the retinal polyene chain [17]. The 1741 (-)/1735 (+) cm^{-1} pair is assigned to carboxyl group stretching of D115, which interacts with D96 [18]. In the M state, proton transfer from the deprotonated Schiff base to D85 produces a positive peak at 1760 cm^{-1} from protonated D85 [18], which persists into the N state. Additionally, the enhanced negative peak at 1742 cm^{-1} in the N state indicates proton transfer from D96 to the Schiff base, representing reprotonation of the Schiff base [6].

Significant changes were observed in M145FL33-bR compared with RCL33-bR. The appearance of a vibrational peak at 1762 cm^{-1} in the L state, marking D85 protonation and M-state formation, indicates that the M145F mutation causes direct K→M transition in the bR photocycle. Previous studies have reported that W182 in bR interacts with the methyl group at C13 of the retinal side chain; during the K-to-L transition, W182 moves toward M145 and the L93 side chain rotates, reducing the distortion of 13-cis retinal [14]. The M145F mutation may hinder W182 movement toward M145 and prevent L93

side chain rotation. Crystal structures of aR2 and cR3 (cruxrhodopsin-3) reveal that F150 or L149 at the position corresponding to M145 restricts C13 methyl movement of retinal, blocking K-to-L conversion; aR2 shows loss of the L state [19], while cR3 shows accelerated L decay that is undetectable kinetically [20]. Therefore, we propose that the M145F mutation increases local steric hindrance, affecting L-state formation and accelerating its decay, thereby converting the K→L→M transition to direct K→M transition, a phenomenon consistent with that observed in RCL33-aR4.

Compared with RCL33-bR, the infrared spectra of RCL33-aR4 intermediates show significantly weakened C=C stretching vibration peaks of retinal, with difference spectra showing smaller differences between the two retinal states. Moreover, like M145FL33-bR, RCL33-aR4 shows D86 protonation in the L state (appearance of positive peak at 1765 cm^{-1}).

Compared with RCL33-aR4, the F146ML33-aR4 mutant shows essentially no change in the protonation state of aspartic acid residues. Both D86 and D97 are protonated in the L state (1766 cm^{-1} (+), 1742 cm^{-1} (-)), indicating that the F146ML33-aR4 mutation does not significantly affect the protonation process or intermediate state transitions.

The C=C stretching vibration peak shape of the F146ML33-aR4 mutant retinal polyene chain is similar to that of RCL33-bR, with comparable intensity, while the peak intensities from C=C stretching vibrations in the intermediate difference spectra of RCL33-aR4 and M145FL33-bR are relatively weak. This may be because the methionine-to-phenylalanine mutation increases steric hindrance, while the phenylalanine-to-methionine mutation decreases it. Therefore, RCL33-aR4 and M145FL33-bR, having similar steric hindrance, show little effect on retinal C=C stretching vibrations with no significant changes in difference spectra. Conversely, F146ML33-aR4 and RCL33-bR show opposite effects, where steric hindrance significantly impacts C=C stretching vibrations. Although the F146ML33-aR4 mutation does not significantly affect intermediate state transitions, consistent with RCL33-aR4, it does influence retinal C=C stretching vibrations, indicating that while the mutation reduces steric hindrance and makes the spatial structure more flexible, allowing more flexible C=C vibrations, it does not hinder the original overall movement trajectory of residues related to the mutation site and retinal.

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Note: Figure translations are in progress. See original paper for figures.