Structural basis of diversification of fungal retinal proteins probed by site-directed mutagenesis of *Leptosphaeria* rhodopsin

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Abstract Numerous fungal genomes encode homologs of bacteriorhodopsin (BR), but only two fungal rhodopsins were overexpressed and characterized spectroscopically, *Neurospora* rhodopsin (NR) is a slow-cycling sensory rhodopsin-like protein, while *Leptosphaeria* rhodopsin (LR) is a BR-like proton pump. Recently, we found that a conservative replacement of the cytoplasmic proton donor Asp150 by Glu converts LR into an NR-like protein. In this work, we search for structural reasons for the dramatic differences in their photochemistry by mutating the hydrogen-bonding partner of Asp150, and that the dramatic effect of the D150E mutation is not a simple result of the size mismatch. We conclude that while these residues may contribute to the differences between LR and NR, they are not crucial for the optimization of the Schiff base reprotonation (Fig. 1). The D150E mutation affects the reprotonation rate of the Schiff base of LR, rendering the cytoplasmic proton donor as inefficient as in the case of unprotonatable Asn. Additionally, it perturbs the environment of the retinal affecting its conformation and retinal–protein interactions. On the other hand, the D150E-mediated conversion of LR into an NR-like protein was not full, as the strongly bound water in the Schiff base region was preserved in this mutant, making it more similar to BR and LR than to NR [14]. Thus, there should be additional structural contributions to the differences between LR and NR, which we try to identify in this work by comparing amino acid sequences of LR, NR, and other microbial rhodopsins and mutating the selected residues (Thr233, Asp248, and Gly271). To test whether the dramatic effect of the conservative replacement of Asp150 is caused by the size mismatch with its hydrogen-bonding partner, Thr87, the latter was replaced by Ser in order to create more space for a bulkier Glu sidechain. Time-resolved spectroscopic (visible and infrared) characterization of the produced mutants combined with the sequence comparisons suggest that the tested residues are not crucial for the optimization of the Schiff base reprotonation by Asp150, and that the dramatic effect of the D150E mutation is not a simple result of the size mismatch.

Keywords: Retinal protein; Microbial rhodopsin; Proton transport; Photosensory receptors

1. Introduction

Recent genome sequencing projects brought enormous wealth of information on the new homologs of bacteriorhodopsin (BR). These microbial rhodopsins are found in hundreds of eubacterial species and dozens of microscopic fungi and algae. While some microbial rhodopsins preserve structures and functions similar to those of their halorhodopsin counterparts, some diverged and acquired new functions [1–4]. In fungi, the exact physiological role of rhodopsins has not been established. Even though fungal rhodopsins conserve most of the retinal-binding pocket and proton-conducting semichannels of BR [3–7], a detailed bioinformatic analysis shows heterogeneity of this group implying possible functional diversification [1,2]. This is in line with the recent results of heterologous overexpression and biophysical characterization of two fungal rhodopsins, from *Neurospora crassa* (NR) and *Leptosphaeria maculans* (LR), which showed very different photochemical behaviors [8–10]. NR possesses a slow cycle of photochemical transformations, similar to that of sensory rhodopsin II of halorhodopsin, and does not pump protons [8,9,11], but LR is a fast-cycling protein capable of the light-driven proton translocation, similar to BR [10]. Consistent with these results, low-temperature Fourier-transform infrared (FTIR) showed the absence of strongly bound water, believed to be a signature of the proton-transporting rhodopsins, in the active center of NR, but detected such water molecule in LR [12,13]. The structural basis of the functional difference between these similar proteins was not clear. Recently, we found that a conservative replacement of Asp150, the cytoplasmic proton donor of the Schiff base homologous to Asp96 of BR, converts LR into an NR-like protein [14]. Asp150 was replaced by Glu because NR has Glu42 at the homologous position (Fig. 1). The D150E mutation affects the reprotonation rate of the Schiff base of LR, rendering the cytoplasmic proton donor as inefficient as in the case of unprotonatable Asn. Additionally, it perturbs the environment of the retinal affecting its conformation and retinal–protein interactions. On the other hand, the D150E-mediated conversion of LR into an NR-like protein was not full, as the strongly bound water in the Schiff base region was preserved in this mutant, making it more similar to BR and LR than to NR [14]. Thus, there should be additional structural contributions to the differences between LR and NR, which we try to identify in this work by comparing amino acid sequences of LR, NR, and other microbial rhodopsins and mutating the selected residues (Thr233, Asp248, and Gly271). To test whether the dramatic effect of the conservative replacement of Asp150 is caused by the size mismatch with its hydrogen-bonding partner, Thr87, the latter was replaced by Ser in order to create more space for a bulkier Glu sidechain. Time-resolved spectroscopic (visible and infrared) characterization of the produced mutants combined with the sequence comparisons suggest that the tested residues are not crucial for the optimization of the Schiff base reprotonation by Asp150, and that the dramatic effect of the D150E mutation is not a simple result of the size mismatch.

2. Materials and methods

2.1. Expression of LR mutants in *Pichia pastoris*

The N-terminally truncated D150E, T87S, T87S/D150E, T233A, D248G, and G271P mutants of LR were produced by derivatization of the wild-type construct in pHIL-S1 vector as described previously.
exception was the T233A mutant, where His +Muts (slow utilization of idly), and proper integration was checked by the colony PCR. The only (by their ability to grow without histidine and to utilize methanol rap-

Fig. 1. (A) Partial alignment (manually edited CLUSTALW) of primary sequences of conserved transmembrane regions of BR, LR, and NR. Residues conserved in all variants of BR [15,16] are shaded, mutated residues shown by arrows. (B) Selected LR residues mapped on the structure of the expressed part of the protein (from residue 49) modeled on the BR template (PDB coordinates 1C3W) using Swiss-Model and Swiss-PDBViewer.

After the transformation by electroporation of the GS115 strain of Pichia pastoris according to the invitrogen Pichia expression kit manual, the transformants were screened for His’Mut’ phenotypes (by their ability to grow without histidine and to utilize methanol rapidly), and proper integration was checked by the colony PCR. The only exception was the T233A mutant, where His’Mut’ (slow utilization of methanol) phenotypes were selected as having better expression of the protein. The expression of LR was induced by methanol and the cells were grown in the presence of exogenous all-trans-retinal as before [12].

When the expression levels were insufficient to select high-expression transformants visually, based on the intensity of red color of the cells (the case of the T87S and T233A mutants), we used Western blot and SDS–PAGE (Bio-Rad) screening to detect the His-tagged products of the M intermediate. The interferogram acquisition time is followed by incubation with the primary antibody (1:5000 dilution) for 1 h at room temperature or overnight at 4°C. After the blocking, the membrane was rinsed by TBS-T buffer (5 min, 3x) followed by incubation with the primary antibody (1:5000 dilution) for 1 h at room temperature or overnight at 4°C. After incubation with the secondary antibody (1:10000 dilution, 1 h), the membrane was washed with SDS–TBS-T buffer (5 min, 3x).

2.2. Isolation and purification of LR for spectroscopic studies

The detailed procedures for making samples for FTIR and visible spectroscopy are given elsewhere [10,12–14]. Briefly, DM-washed LR-containing membranes of P. pastoris were encased in polyacrylamide gels for visible spectroscopy. For FTIR measurements, the membranes were solubilized in Triton X-100, LR was batch-purified on Ni2+-NTA resin (Qiagen) and reconstituted into preformed 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyristoyl-sn-glycero-3-phosphate (DMPC/DMPA) (9:1) liposomes by removal of the detergent with Bio-Beads SM-2 (Bio-Rad). The hydrated films of the liposomes were deposited between two CaF2 windows and sealed. Time-resolved spectroscopic characterization of the photocycles in the visible (home-built flash-photolysis) and infrared (Bruker IFS66v rapid-scan FTIR) ranges was performed as described elsewhere [10,17], using green laser (353 nm, 7 ns) as the excitation source.

3. Results and discussion

3.1. Testing the nature of the D150E contribution to the LR/NR differences

Recently, we demonstrated that a replacement of the cytoplasmic proton donor of the retinal Schiff base, Asp150, by Glu converts LR into a slow-cycling NR-like protein [14]. The presence of Glu142 in NR at this position, homologous to that of Asp96 of BR (Fig. 1), suggests that the exact nature of this residue may control photochemistry of fungal rhodopsins, but the reason for such dramatic effect of the conservative Asp/Glu replacement is not clear. One possible explanation is that a larger Glu150 sidechain causes a perturbation of the protein structure in the D150E mutant of LR and/or has improper proton affinity (pKa) to serve as a proton donor for the Schiff base. Thus, we replaced Thr87, the putative hydrogen-bonding partner of Asp150 homologous to Thr46 of BR, with a smaller Ser, in hope to accommodate a larger sidechain of Glu and revert the effect of D150E mutation.

Even though the reprotonation of the Schiff base in D150E is as slow as in D150N, Glu150 may still serve as its proton donor. To test this, one has to accumulate the N intermediate (monitored by FTIR bands of 13-cis-retinal at 1188 cm−1, Fig. 2), which is difficult due to the extremely slow decay of the preceding M intermediate. We have accelerated the M decay by decreasing pH to 5 and adding sodium azide and found that slow deprotonation of Glu150 indeed occurs during the M → N transition as shown by the appearance of the new

![Fig. 2. Rapid-scan FT-IR difference spectra after photoexcitation of the D150E mutant of LR in DMPC/DMPA liposomes. The protonated carboxylic acid stretches and retinal C–C stretches are given elsewhere [10,12,17]. The interferogram acquisition time is 12 ms.](image-url)
negative 1718 cm\(^{-1}\) vibrational band (Fig. 2) absent in the wild-type spectra (not shown) and assigned to this residue earlier [14]. The large positive bands appearing at higher frequencies should correspond to protonation of Asp139 (the homolog of Asp85 of BR) and perturbation of Asp169 (the homolog of Asp115 of BR) as suggested earlier [10,14].

If Glu150 is still protonated in the M intermediate, its pK\(_a\) can be improperly modulated by Thr87, which was tested in the double T87S/D150E mutant. The photocycles of the T87S/D150E and D150E mutants are compared in Fig. 3. It is clear that, contrary to the expectations from introducing a smaller sidechain, the second mutation is not efficient in reverting the D150E mutation. Instead, it exaggerates its phenotype making the M decay even slower than in D150E in the wide pH range (only pH 7 is shown), similar to the situation observed in the T46V/D96N mutant of BR [18]. To test the effect of T87S replacement in the wild-type background, we studied the photocycle of the single mutant (Fig. 3) and found that in the case of Asp150 (wild-type background) this mutation seems to decrease the pK\(_a\) of the carboxylic proton donor resulting in a faster M decay and slower N decay. Accordingly, we have detected higher accumulation of the N intermediate by FTIR (not shown) and by the tail of negative absorption change at 560 nm (Fig. 3), similar to that of the T46V mutant of BR [18]. Thus, the T87S replacement produced the desired acceleration of the Schiff base reprotonation in the case of Asp150 (wild-type) but not in the case of Glu150 (D150E).

These results imply that a larger size of the carboxylic sidechain may not be the only reason for the defective phenotype of D150E, as it can not be reverted by simply making more space at this site. This is unexpected, as bioinformatic analysis of the sequences of bacterial rhodopsins suggests that if a homolog of D96 is Asp, it is paired with Thr, as in the case of BR and its haloarchaeal homologs [15,16], while eubacterial proton pumps, such as proteorhodopsins (PR), xanthorhodopsin, and *Gloeobacter* rhodopsin (GR) demonstrate homologous Glu/Ser pair [2,19,20]. On the other hand, eubacterial rhodopsins PR [21] and GR (M. Miranda et al., unpublished) are tolerant to the Glu/Asp replacement, which does not create serious changes in the photocycle. Additionally, while all LR-like fungal rhodopsins [2] demonstrate Asp/Thr match and most of NR-like rhodopsins show Glu/Thr size mismatch (or do not have the carboxylic residue at all), there is an example of an NR-like protein from *Gibberella zeae*, which has the Glu/Ser pair (Table 1).

Thus, all of the above suggests that if the apparent Glu/Thr size mismatch is the source of the slow reprotonation of the Schiff base in NR and D150E LR, its mechanism is not solely based on the bulk disparity. As Glu150 is protonated in the ground state of LR, as was shown by low-temperature FTIR [14], the introduction of the negative charge can not be the cause either. As we observed that the deprotonation of Glu150 occurs, albeit slowly, in the M–N transition (Fig. 2), the most probable cause is the improper modulation of the pK\(_a\) of this carboxylic acid in the photocycle, which may result from unfavorable stereochemical conditions for the intercalation of a water molecule, as observed in the late M state of BR [22].

![Fig. 3. Kinetics of the light-induced absorption changes in the LR variants: (A) wild-type, (B) D150E, (C) T87S/D150E, (D) T87S. The data for (A) and (B) are taken from our earlier work [10,14] for comparison. Conditions: gel-encased *P. pastoris* membranes equilibrated with 0.1 M NaCl, 0.05 M sodium phosphate, pH 7, temperature 22°C. Blue – 420 nm, green – 560 nm, red – 620 nm. Difference in the signal amplitudes and noise levels reflects different levels of the expression.](image_url)
3.2. Searching for additional contributions to the LR/NR differences

Previous analysis of the differences in primary structures of LR and NR [2,13] showed that there are very few alterations in the residues considered to be important for BR. Thus, we looked for more subtle changes and have identified three extra residues (Fig. 1), which are different between NR and LR and could potentially contribute to the differences in their photo-

Table 1

<table>
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<tr>
<th>Species</th>
<th>Homolog of T46 of BR</th>
<th>Homolog of D96 of BR</th>
<th>Homolog of T178 of BR</th>
<th>Homolog of E194 of BR</th>
<th>Homolog of V217 of BR</th>
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<th>Homolog of E194 of BR</th>
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a The data were obtained by BLAST searches of public sequence databases and CLUSTALW alignments, and classified as described elsewhere ignoring the ORP-like sequences [2,7]. The sequence from Podospora was excluded from the comparison as being transitional between the two classes.

Fig. 4. Kinetics of the light-induced absorption changes in the LR variants: (A) wild-type (B) T233A, (C) D248G, (D) G271P. The data for (A) are taken from our earlier work [10] for comparison. Conditions: gel-encased P. pastoris membranes equilibrated with 0.1 M NaCl, 0.05 M sodium phosphate, pH 7, temperature 22 °C. Blue – 420 nm, green – 560 nm, red – 620 nm. Difference in the signal amplitudes and noise levels reflects different levels of the expression.
chemistry. First, Thr233 of LR, the homolog of Thr178 of BR, is replaced by Ala in NR. According to the X-ray structures of BR and its M state, Thr178 is located in a structurally hot region between Asp96 and Trp182 and changes its hydrogen-bonding in the M state [22]. Another candidate located in this region is Gly271, the homolog of Val217 of BR, represented by Pro in NR. Val217 of BR is located next to the retinal-binding Lys in the so-called π-bulge, which changes its structure in the M state [22], and it was suggested that homologous Pro in NR can affect the reprotonation switch of the Schiff base [9]. Finally, Asp248, the homolog of Glu194 of BR, is changed into Gly in NR. Glu194 is a part of the extracellular proton-releasing complex of BR, and its replacement has long-range effects on the Schiff base region [23].

We measured the photocycle kinetics for the T233A, G271P, and D248G mutants of LR in the wide pH range, and found that the phenotypes of these mutants do not significantly differ from the wild-type (pH 7 data, Fig. 4, other pH values not shown). Slight (2–3-fold) deceleration of the M decay in the D248G mutant is similar to that observed in the homologous mutant of BR [23]. Thus, at the present state, Asp150 remains the only major determinant of the fast BR-like photocytchemistry of LR, as its D150E mutant perfectly emulates the M state [22], and it was suggested that homologous Pro in NR-like and LR-like fungal rhodopsins (Table 1). The situation is quite different for the T233A and D248G mutations. Even though Thr178 of BR (Thr233 of LR) is not conserved between different halobacterial species [15,16], it is strictly conserved for LR-like fungal rhodopsins and is present as a smaller Ala or Ser in NR-like proteins (Table 1). Likewise, Glu-194 of BR (Asp-248 of LR) is present as a carboxylic acid in all LR-like proteins, but is a non-protonatable residue in NR-like proteins. It follows that while these residues may contribute to the differences between LR and NR, they do not participate in the optimization of the Schiff base reprotonation by Asp150.

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