Mimicking Microbial Rhodopsin Isomerization in a Single Crystal

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Supporting Information

ABSTRACT: Bacteriorhodopsin represents the simplest, and possibly most abundant, phototropic system requiring only a retinal-bound transmembrane protein to convert photons of light to an energy-generating proton gradient. The creation and interrogation of a microbial rhodopsin mimic, based on an orthogonal protein system, would illuminate the design elements required to generate new photoactive proteins with novel function. We describe a microbial rhodopsin mimic, created using a small soluble protein as a template, that specifically photoisomerizes all-trans to 13-cis retinal followed by thermal relaxation to the all-trans isomer, mimicking the bacteriorhodopsin photocycle, in a single crystal. The key element for selective isomerization is a tuned steric interaction between the chromophore and protein, similar to that seen in the microbial rhodopsins. It is further demonstrated that a single mutation converts the system to a protein photoswitch without chromophore photoisomerization or conformational change.

INTRODUCTION

The conversion of light into chemical energy is an essential natural process. The key phototrophic reaction is performed via exquisitely evolved chromophore-bound protein complexes.1 The retinal-bound opsins, known as rhodopsins, are the earliest such proteins known,2 exhibiting functional diversity that includes proton and ion pumps, ion channels,3 signaling,4 vision,5 and sensing.6 The apo-proteins, referred to as opsins, are such proteins known,2 exhibiting functional diversity that includes proton and ion pumps, ion channels,3 signaling,4 vision,5 and sensing.6 The apo-proteins, referred to as opsins, are

Figure 1. All-trans retinylidene chromophore of bacteriorhodopsin photoisomerized to the 13-cis isomer followed by thermal regeneration of the all-trans isomer.

leading to the translocation of protons across the membrane.1b,7 This is in contrast to the chromophore’s solution behavior, where photon absorption leads to a wide distribution of retinal isomers.8 A vast literature that includes time-resolved spectroscopy, structural biology, most notably X-ray crystallography,9 and biochemical experiments10 describes the structure, spectroscopy, and lifetimes of the various bacteriorhodopsin intermediates and identifies the key protein residues essential for function.1b,9a,11 Nonetheless, fundamentally, the function of all rhodopsins depends on the specific bond isomerization of the bound retinylidene chromophore. Therefore, the creation of an orthogonal, simplified protein system, amenable to routine biophysical measurements, especially atomic-resolution structural interrogation, that recapitulates a specific bond isomerization similar to that seen in bacteriorhodopsin provides a unique opportunity for future investigations.

We have had a long-standing interest in using protein mimics to understand protein–chromophore interactions that lead to wavelength regulation in rhodopsins.12 We now turn our attention to the factors that govern the isomerization events by investigating the minimal requirements of specific isomerization in a model system that mimics the hallmarks of the natural system. Herein, we report the re-engineering of human cellular retinoic acid binding protein II (CRABPII), a small, soluble β-sandwich cytosolic protein from the intracellular lipid binding protein family, to undergo specific photoisomerization of all-trans to 13-cis retinylidene and thermal isomerization back to the all-trans isomer in a single crystal, representing a complete photocycle of the microbial rhodopsins. Serendipitously, with a specific mutant, we observe a case of protein conformational...
change in response to photoexcitation at a wavelength in which protein amino acids do not absorb. This is in lieu of the expected chromophore isomerization, signifying the response of the protein to the chromophore excited state. This study establishes a new model system for studying the isomerization pathway of retinoids in real time, with well-characterized starting and ending points of isomerization.

■ RESULTS AND DISCUSSION

Choosing the Initial Protein Template. To initiate our investigation, we required a platform that would support photoinduced isomerization of the bound chromophore. As described in our previous studies, a number of CRABPII variants exhibited photoinduced changes in the protonation state of a retinylidene-bound protein, which ultimately dictated the pigmentation of the protein retinylidene-bound complex.12e X-ray crystallography and UV–vis spectroscopy showed that imine bond isomerization from 15-syn to 15-anti is responsible for this change. As depicted in Figure 2a, CRABPII hexamutant M1 (R111K:Y134F:T54V:R132Q:P39Y:R59Y) bound to retinal. The photoinduced isomerization of the imine functionality leads to changes in its pKa, and consequently its protonation state. Hydrophilic and hydrophobic environments of the imine nitrogen atom for the cis and trans geometry, respectively, are highlighted in the figure obtained from the crystal structure of each form (PDB IDs 4YFP and 4YFQ). (b) The present study aims to induce photoisomerization to the C13 isomer while maintaining the protonated iminium. (c) All-trans-retinal-bound M1-CRABPII, with key residues highlighted.

![Figure 2](image-url)
Solution Photoirradiation. Intrigued by the significant drop in the absorption of both SB and PSB bands upon irradiation of the M1-L121E/all-trans-retinal complex with either UV or green light (Figure 3a), the chromophore was extracted from photoirradiated samples and analyzed by HPLC (Figure S6). Three peaks were apparent in the HPLC traces for all reactions: photolysis with a UV band-pass filter (300–400 nm) and a blue band-pass filter (440 nm ± 20 nm); green light irradiation with a band-pass filter (>500 nm) and a 399 nm laser. In the extracts of all four reactions, the peak that coelutes with authentic all-trans retinal was major, while the second peak coeluted with authentic 13-cis retinal and the third peak was assigned as 9,13-di-cis-retinal, based on the 1H NMR aldehyde chemical shift comparison with the reported isomer. (See the SI and Figures S6–S8 for details of each extract and the ratio of identified isomers.) The extinction coefficient of cis isomers is approximately 80% of that of the all-trans isomer, thus the drop in absorption noted above is partially attributed to the production of the 13-cis and 9,13-di-cis-isomers, along with some potential photobleaching of the chromophore. Nonetheless, the production of the 13-cis isomer was exciting, although HPLC analysis of the extracted chromophores, the electron density of the bound chromophore, after 5 min of laser irradiation, shows no evidence of all-trans retinal and instead is consistent with its quantitative photoconversion to the 13-cis-15-syn isomer (Figure 3d; see Figure S11 for the crystal structure of the 30 s irradiated sample). The observed photoisomerization of the bound retinylidene from 13-trans-15-anti to 13-cis-15-syn is similar to the "bicycle pedal mechanism" as first proposed by Warshel and also suggested for the dark adaptation of bacteriorhodopsin. In his proposal, two adjacent double bonds undergo isomerization, as seen with the photoisomerization of M1-L121E. Although it is not possible to rule out different mechanisms with end-state observations from crystal structures, the final geometry of the photoisomerized M1-L121E is in contrast to the "hula-twist" proposal that predicts the isomerization of an olefin and its adjacent single bond. An alternate explanation for the formation of the 15-syn isomer could be the fast thermal adaptation of 15-anti to 15-syn, which is not detectable in the crystal within the time resolution of our method. As shown in Figure 3e, the overlay of prelaser and postlaser irradiation indicates substantial β-ionone ring rotation and polyene movement. Furthermore, the conformational changes in Glu121, which follows the movement of the imine nitrogen atom, and in Lys111 are apparent. Interestingly, the position of Tyr39 remains fixed before and after irradiation. Tyr39 may act as a "steric pillar" that dictates the path of isomerization in a specific direction (Figure 3e). The role of Tyr39 mimics that of Trp86 in bacteriorhodopsin, which sterically packs against the retinal and assists in directing the isomerization (Figure 3).
photoisomerization of the C13–C14 double bond.16 As shown in the overlay of M1-L121E and bacteriorhodopsin17 (Figure 3f), Glu121 and Tyr39 occupy a similar space relative to the retinal, similar to Trp86 in bacteriorhodopsin. Once again, the fortuitous yet critical role of the L121E mutation is apparent because it aids in orienting Tyr39 in a conformation that resembles Trp86 in bacteriorhodopsin, leading to specific 13-cis photoisomerization. To verify the importance of the steric buttressing effect of the P39Y mutation, it was mutated to Gln, resulting in M1-L121E:P39Q. (The equivalent P39F mutation gave uninterpretable results.) As anticipated, there is no evidence of isomerization to the 13-cis isomer either in solution or in the crystal (Figures S12 and S13), thus confirming the critical function of the tyrosine residue. These observations, however, cannot disregard electronic effects as a result of mutations such as P39Y, where the polarity of its immediate environment has changed. It is noteworthy that the isomerization in the crystalline state of M1-L121E/all-trans-retinal complex indicates complete conversion to the 13-cis isomer, suggesting the mixture obtained in solution to be the result of postirradiation thermal relaxation. Nonetheless, we cannot rule out the possibility that the difference in behavior of crystal vs solution-state isomerization could be due to the solid-state environment where the lattice interaction restricts the conformational flexibility of molecules.

Thermal Reversion to Original State. With photoisomerization to 13-cis confirmed, its thermal stability was next investigated. To this end, photochemically generated 13-cis-bound M1-L121E crystals were left in the dark at room temperature for 10 and 25 min. We anticipated the thermal reversion of the 13-cis isomer to the apparently more thermodynamically stable all-trans isomer in M1-L121E. This would complete the photocycle that is essentially identical to that of the microbial rhodopsins, though on a significantly slower time scale. Structures of all of the thermally equilibrated crystals (after 10 and 25 min of equilibration) revealed electron density consistent with all-trans-retinal bound M1-L121E, confirming the completion of a microbial rhodopsin photocycle (Figures 4a and S14–S16). The main characteristics of the structure, most notably the rotation and trajectory of the β-ionone ring, are clearly observed (Figure S16). This is consistent with the fact that the extraction of retinal-bound M1-L121E, after irradiation with visible light in solution, shows both all-trans (Figures S6 and S8) and 13-cis isomers, with the latter the result of irradiation and the former the result of thermal relaxation to the more stable all-trans form. Thus, a complete photocycle, analogous to a microbial rhodopsin, has been generated in an orthogonal protein and confirmed at atomic resolution.

Mechanistic Insights and Predictions. Our interpretation of the results described above naturally leads to two predictions regarding the mechanism. First, stabilizing or restricting the ionone ring conformation can stabilize distinct conformational states (either all-trans or 13-cis) of retinal. Second, both Glu121 and Tyr39 should be essential in giving a 13-cis isomerizing system. On the basis of the first prediction, we hypothesized that the stabilization of the rotated ionone ring conformation would also stabilize the binding of the 13-cis isomer in the binding pocket (Figure 3e). To this end, Ala32, located at the mouth of the binding pocket, was mutated to aromatic amino acids to persuade the rotation of the ring as a result of steric compression afforded by the larger amino acids. The incubation of 13-cis-retinal with M1-L121E:A32Y leads to an absorption indicative of an iminium. (See the UV–vis trace in Figure S17b.) Gratifyingly, the crystal structure of the latter complex (with 1.58 Å resolution) clearly shows the 13-cis isomer bound exclusively in the binding cavity (Figure 4b,c). The overlay of this structure with M1-L121E postirradiation (13-cis-isomer) shows both the β-ionone and polyene moieties to be quite similar (Figure S17), while the overlay of this structure with all-trans retinal-bound structures shows clear and obvious structural variations (Figure 4b). An analysis of the high-resolution structure indicates that the 13-cis isomer is stabilized by the packing of several aromatic residues, including Phe15, Tyr39, Tyr32, Tyr59, and Trp109 inside the binding pocket, mostly through “aromatic sandwiches” (Figure 4c). Interestingly, this is reminiscent of aromatic/chromophore interactions observed in a number of microbial rhodopsins,16,64,17 where the chromophore finds itself well-packed in a sphere of aromatic residues (Figure 4d). These results demonstrate that by increasing the pKₐ and packing of P39Y closer to those of the polyene (using the L121E mutation) and altering the β-ionone ring packing (via the A32Y mutation), a 13-cis “unfriendly” binding pocket (M1) is converted to a 13-cis-specific protein, nestling a well-ordered 13-cis retinylidene inside the binding pocket.

To test the second prediction, mutations were made to both Tyr39 and Glu121 residues. As described above, the P39Q mutation to give M1-L121E:P39Q resulted in a protein complex incapable of isomerizing the C13 double bond. This demonstrates the importance of the steric interaction between Tyr39 and the chromophore in directing isomerization to the 13 bond. To verify the role of the L121E mutation in the iminium stabilizing species, we examined the photochemical characteristics of the structurally conservative M1-L121Q mutant. As
shown in Figure 5a, the irradiation of M1-L121Q in solution leads to behavior similar to that of mutants that undergo imine isomerization with two apparent pKₐ’s for each isomer. UV light irradiation (<400 nm) results in a loss of intensity in the 374 nm SB absorption, concomitant with a large increase in the PSB absorption at 522 nm. Conversely, irradiation with visible light (>500 nm) reduces the PSB absorption at 522 nm along with an increase in the 374 nm SB absorption (Figures S18 and S19). At first, this result was not unexpected because the loss of the salt bridge afforded through the interaction of the iminium with Glu121 could reestablish the imine cis/trans isomerization observed before, leading to two states with distinct pKₐ’s (Figure S20). Nonetheless, the crystal structure analysis reveals a surprising and unanticipated mechanism for the observed pKₐ change.

**Photoirradiation Leads to Side-Chain Motion.** Not unexpectedly, all-trans retinal-bound M1-L121Q is structurally almost identical to all-trans retinal-bound M1-L121E, with Gln121 making a direct hydrogen bond with the iminium nitrogen atom (Figure Sb). Surprisingly, the irradiation of the all-trans bound crystal with the 399 nm laser showed no evidence of chromophore isomerization or conformational change. Instead, Gln121 adopts two conformations: the original conformation seen in the dark (55% occupancy) and a new position that is rotated away from the iminium (45% occupancy), resulting in a naked imine nitrogen atom with no other interacting residues (Figure S21b). Interestingly, irradiation with green light (532 nm laser irradiation) leads to the complete movement of Gln121 away from the imine nitrogen atom (Figures Sb and S21c). An imine under these circumstances would be expected to have a substantially suppressed pKₐ relative to the dark state, consistent with the solution result that shows an increase in the SB form upon light irradiation (Figure 5a).

In contrast to the expected isomerization of the chromophore’s double bond, leading to a change in the environment of the imine and its pKₐ, irradiation of the M1-L121Q retinal complex leads to a change in the conformation of an interacting amino acid side chain, thus effectively altering the environment. The mechanism of this process is unclear, but one possibility is that the photoinduced motion of the chromophore induces the conformational change in the side chain.

**CONCLUSIONS**

An artificially designed protein that recapitulates the photocycle of microbial rhodopsins, specifically and quantitatively photos-isomerizing all-trans-retinal to 13-cis-retinal in a well-diffracting crystal, has been created. Because of its slow rate of isomerization, our engineered rhodopsin mimic can be exploited using relatively simple time-resolved spectroscopic and crystallographic methods. It also introduces an ideal system for the design and study of photoactive and photo-switchable protein systems.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b12493.

Experimental data including protein expression and purification, UV–vis spectra, HPLC extraction, crystallization conditions, crystal irradiation, and X-ray data collection and refinement statics; crystallographic files such as atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes: 6MOP, 6MQZ, 6MQY, 6MQW, 6MQX, 6MPK, 6MQQ, 6MQR, 6MOV, 6MQI, 6MQJ, 6MOX, and 6MR0) (PDF)

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ABBREVIATIONS

7TM, seven transmembrane helices; SB, Schiff base; CRABPII, human cellular retinoic acid binding protein II; PSB, protonated Schiff base; BLUF, blue light using FAD; HPLC, high-pressure liquid chromatography; M1, R111K;Y134F;T54V;R132Q;P397Y;R59Y

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