Photochemical and Thermal Stability of Green and Blue Proteorhodopsins: Implications for Protein-Based Bioelectronic Devices

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The photochemical and thermal stability of the detergent-solubilized blue- and green-absorbing proteorhodpsins, BPR and GPR, respectively, are investigated to determine the viability of these proteins for photonic device applications. Photochemical stability is studied by using pulsed laser excitation and differential UV−vis spectroscopy to assign the photocyclicity. GPR, with a cyclicity of 7×10^4 photocycles protein^{-1}, is 4−5 times more stable than BPR (9×10^3 photocycles protein^{-1}), but is less stable than native bacteriorhodopsin (9×10^5 photocycles protein^{-1}) or the 4-keto-bacteriorhodopsin analogue (1×10^5 photocycles protein^{-1}). The thermal stabilities are assigned by using differential scanning calorimetry and thermal bleaching experiments. Both proteorhodopsins display excellent thermal stability, with melting temperatures above 85 °C, and remain photochemically stable up to 75 °C. The biological relevance of our results is also discussed. The lower cyclicity of BPR is found to be adequate for the long-term biological function of the host organism at ocean depths of 50 m or more.

Bioelectronics seeks to apply the unique functionalities of biological molecules in electronic or photonic device applications. Promising technologies under investigation include nanodisk arrays,[1] DNA-stabilized quantum-dot light-emitting diodes,[2] biotemplated nanowires,[3] and protein-based memories and photonic devices.[4,5] Bacteriorhodopsin (BR), the most fully characterized type I retinylidine protein, has long been studied for device applications due to unique photophysical properties and excellent stability.[4,6] Many BR-based applications capitalize on the structural and spectral properties of the molecule, which include the following: a self-assembling 2D semicrystalline lattice,[7] a high photochemical cyclicity,[4,6] a fast photocycle involving discrete spectral signatures throughout the visible spectrum,[8] a high thermal stability,[9,10] and a long-lived branched-photocycle intermediate.[11,12] For an excellent review of optical memories based on BR, see ref 13.

Proteorhodopsin (PR), a eubacterial photoactive membrane protein,[14] displays many photophysical similarities to BR.[15−19] These similarities have prompted interest in the potential use of PR in bioelectronic and biophotonic devices, an interest that is enhanced by the ability to express large quantities of this protein in *Escherichia coli*.[20,21] Initial investigations of the holographic[22] and photovoltaic[15,23] properties of PR and applications of PR in security inks,[24] binary optical memories,[25] and solar cells[26] have been reported. A majority of these applications follow logically from previous BR-based devices, due to the similar photocycles of BR and PR (Figure 1). However, PRs offers some comparative advantages in terms of both functionality as well as cost and scale of expression.[24−26]

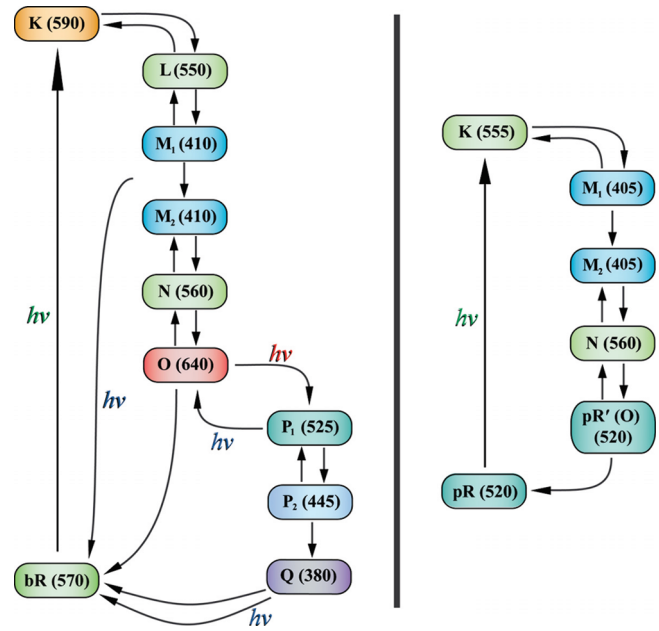

Figure 1. Comparison of the photocycles of native BR (left) and detergent-solubilized green-absorbing proteorhodopsin (right) (see text). Each photointermediate is shown with the respective absorption maximum in parentheses (in nm) at ambient temperature. We note that the pR′(O) intermediate of the GPR photocycle is a complex photostate that is similar to the O state of the BR photocycle. This photostate is not directly observed in visible spectra and can only be identified by kinetic analysis[18] or by FT-IR spectroscopy.[89] No comparable study of the BPR photocycle has been reported.

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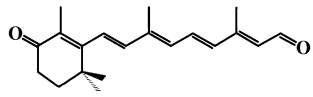
Virtually all protein-based photonic devices are designed to function without routine replacement of the photoactive element and thus the photoactive component must be stable to thermal and photochemical stress imposed by the operating characteristics of the device. Although many PR variants are found throughout the oceans of the Earth,[14,27,28] all identified thus far are classified as either blue-absorbing (BPR) or green-absorbing (GPR) proteins.[27−30] Therefore, the current study is based on an analysis of the most commonly reported homologues of both BPR (Hot75m1)[31] and GPR (EBAC31A08).[32,33] Many of the current biochemical and biophysical studies concerning both PR molecules use *E. coli* expressed protein that is detergent solubilized. We selected octyl-β-D-glucopyranoside (OG), a common detergent for solubilizing both PR molecules,[15,34] for all PR experiments described herein.

Methods and Materials

Protein Preparation and Purification. Proteorhodopsin (6x His tag) was prepared as previously described.[14] All-*trans* retinal, solubilized in ethanol, was added to intact cells during induction of the protein. The protein was purified via a nickel Sepharose column, solubilization in 1% OG and was stored in 100 mM Tris-HCl (pH 8.0) and 0.05% OG. Bacteriorhodopsin was prepared by using the standard method.[35] The BR analog, 4-keto-BR, was generated by replacing the native chromophore, all-*trans* retinal, with 4-keto retinal (shown below):



The synthesis and incorporation of the 4-keto analogue is described elsewhere.[36−38] All protein was stored at 4 °C until use.

Photochemical Stability. We assign the photochemical stability in terms of the cyclicity of the protein.[4,6,39−41] There is no accepted definition of cyclicity, and we adopt a conservative definition that measures how many times the protein can undergo a photocycle before 1/e (~37%) of the protein ensemble has denatured. This definition is useful because most bioelectronic devices can function if ~63% of the protein is still active.[4,6,39−41] Photochemical stabilities of BPR, GPR, BR, and 4-keto-BR were measured at 25 °C using the pulsed-laser configuration shown in Figure 2. Details of the experimental setup, which ensure that the sample is evenly irradiated, are presented in the Supporting Information.

All of the proteins investigated in this study exhibit good photochemical stability, with values between 10^3 and 10^6, and thus the cyclicities must be extrapolated from the experimental data. Experimental determination of the cyclicity without extrapolation is both time and resource intensive. The latter process, if pursued, would require hundreds of laser flashlamps and months of experimental time to photochemically denature these proteins while simultaneously monitoring their spectra. Ergo, a reliable lower limit of the cyclicity can be estimated using eq 1

$$\text{cyclicity} = \frac{0.36788}{-\text{slope}} \qquad (1)$$

where the slope refers to the photochemical denaturation slopes measured in the experiments of Figure 3. The cyclicity calculated using eq 1 will be a lower limit. This observation

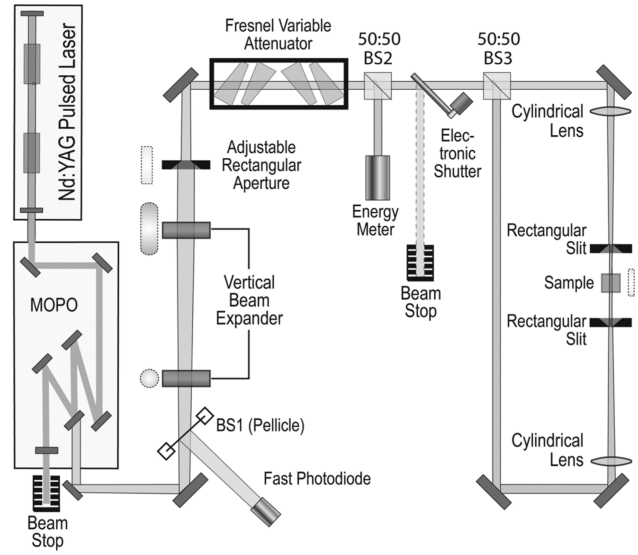


Figure 2. Schematic diagram of the laser apparatus used for measuring the photostability of the light-transducing proteins. The relative shape of the laser beam is shown using dotted lines next to the relevant optical component (see Supporting Information for experimental details).

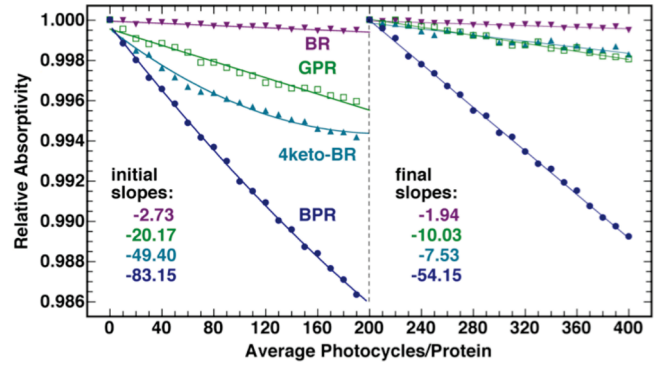


Figure 3. Photochemically induced loss of protein following photoactivation for BR (inverted triangle), 4-keto-BR (triangles), BPR (circles), and GPR (squares) at 25 °C. Each protein exhibits a faster denaturation rate during the first 200 photocycles than during the second 200 photocycles, and the slopes shown are calculated for the 0−200 (initial) and 200−400 (final) segments. The optical density of all samples was renormalized to unity after 200 pulses. All slopes have been multiplied by 10^6. The raw data are shown in Table S1.

follows from recognition that these measurements have denatured only a small fraction of the protein population, and the initial irradiation will selectively remove the less photochemically stable species. The slopes will thus continue to become less negative as the experiment continues. The assumption that the estimated cyclicities will be lower limits is confirmed by the observation that the cyclicity of BR, which is calculated from eq 1 with an assumed slope of -1.94×10^6, yields a cyclicity of only 1.9×10^5. This value is an order of magnitude lower than values reported from other studies.[4,6,39−41] To explore a realistic range of values, the data of Figure 3 was extrapolated from linear to quadratic fits in steps working backward from the final data to include larger fractions of the initial data. The results are shown in Figure 4, and provide a range of values for the cyclicities. The resulting cyclicity ranges are listed on the left-hand side for each of the four proteins and the median cyclicities are summarized in Table 1.

Thermal Denaturation. Differential scanning calorimetry (DSC) studies were carried out using a MicroCal VP-DSC microcalorimeter (Amherst, MA). Samples were prepared using

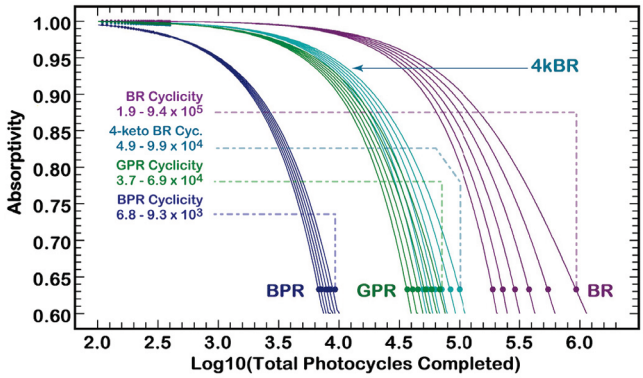


Figure 4. Linear extrapolation of the 200−400 photocycle data from Figure 3 to an absorptivity loss of 1/e ($A = 1 - 1/e = 0.63$) provides an estimate of the photochemical cyclicity of the four proteins at 25 °C. Note that the x axis is in $\log_{10}$ units of the total number of photocycles completed. The cyclicities for BR, 4-keto-BR, GPR, and BPR are listed at left with dashed lines connecting these values to the extrapolated 1 − 1/e absorptivity (single dots).

TABLE 1: Summary of Spectral and Physical Properties for BR, 4-keto-BR, GPR, and BPR

	BR	4-keto-BR	GPR	BPR
λ_{max} (nm)	568	510	525	494
median cyclicity (see Figure 4)	9×10^5	1×10^5	7×10^4	9×10^3
pK_A of the PSB counterion[a]	2.3	n.d.[h]	7.4	7.4
photocycle time (ms)[b]	10	6240	153	n.d.[h]
blue state formation (ms)[c]	0.05	1	0.04	n.d.[h]
blue state decay (ms)[c]	1	5400	0.5	n.d.[h]
red state decay (ms)[d]	8	180	153	n.d.[h]
melting temp (°C)[e]	101	n.d.[h]	89/96[f]	89
photovoltaic signal (mV)[g]	30	n.d.[h]	34	n.d[h]

[a] References for BR,[85] GPR,[30] and BPR.[30] [b] Approximate photocycle lifetimes are measured using native *H. salinarum* lipids for BR[4,36] and 0.2% dodecyl maltoside for GPR.[18] The experimental details are described in the corresponding reference. We note that the BPR photocycle has not been studied in a detergent-solubilized form. When prepared in *E. coli* membranes, however, BPR and GPR exhibit photocylce lifetimes of 150 and 15 ms, respectively.[30] [c] The blue state is defined as the M-state (410 nm) and M-like state (405 nm) for BR and PR, repectively. Different experimental methods, which are described in the references, were used for studying the photointermediate properties of BR,[4] 4-keto-BR,[36] and GPR.[18] [d] The red state is defined as the O-state (640 nm) and N-like state (560 nm) for BR and PR, repectively. Different experimental methods, which are described in the references, were used for studying the photointermediate properties of BR,[4] 4-keto-BR,[36] and GPR.[18] [e] Experimental T_Ms were collected in 50 mM TAPS (pH 8.5) at 1.5 K min^{-1}. [f] The two values represent the two T_Ms observed for GPR. [g] Photovoltaic signals were collected in 5 mM TAPS (pH 8.5).[86] [h] n.d. - not determined

a protein concentration of 1 mg mL^{-1}. All samples and buffers were degassed (8 min; 20 °C) in a MicroCal Thermovac immediately prior to loading. Samples were heated from 30 to 110 °C using 0.25, 0.5, 1.0, and 1.5 K min^{-1} scan rates. At least two consecutive scans were recorded for each sample. The second scan of each sample was transitionless and was used for baseline correction of the initial scan. Analysis of the resulting thermograms was performed using MicroCal Origin 5.0 software. Thermograms were also collected at 2.5 K min^{-1} on a MicroCal VP-capillary DSC. The melting temperature (T_M) was determined from the maximum of the transition of the thermogram. Experimental buffers are described in each figure legend.

The activation energy of denaturation (E_{APP}) was determined from the T_M of the endotherms at each scan rate according to the modified Arrhenius expression:

$$\ln\left(\frac{\upsilon}{T_M^2}\right) = \text{const} - \frac{E_{APP}}{RT_M} \quad (2)$$

where υ is the scan rate (K min^{-1}), T_M is the melting temperature (K) from the DSC thermograms, E_{APP} is the apparent energy of denaturation (kJ mol^{-1}), and R is the gas constant (8.314 J mol^{-1} K^{-1}).[42]

Measurements of the kinetically controlled thermal denaturation process were monitored using methods previously described.[42] Briefly, 10 μL of protein (1.0 mg mL^{-1}) sealed in capillary tubes are heated at fixed temperatures for time increments from 0 to 90 min. Samples were then diluted 1:10 in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) or 50 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS, pH 8.5). Absorption spectra were collected on a Cary 50 UV−visible spectrometer (Varian, Inc., Palo Alto, CA) from 750 to 250 nm at ambient temperature and normalized to 750 nm.

For each temperature, the maximal absorbance was determined at set time intervals. To determine the rate of decay (k), the data were fit to a first-order exponential decay equation

$$A_t = A_\infty + (A_0 - A_\infty)e^{-kt} \quad (3)$$

where A_t is the absorbance at time t, A_∞ is the absorbance at time infinity, A_0 is the absorbance at time zero, k is the thermal decay rate (min^{-1}), and t is the time (min).[42]

Calculations. Calculations and figure generation were carried out by using MathScriptor 1.8.64. This programming environment is available without charge to students and faculty (www.mathscriptor.org). The relevant programs are provided in the Supporting Information.

Results and Discussion

The photochemical and thermal stability of BPR and GPR were studied using the methods and procedures described above. Comparisons were made to the native protein and a chromophore analogue form of BR. The latter two proteins were selected because of their device relevance.[43−46]

Photochemical Stability. The stability of a protein to photochemical switching is normally measured in terms of the number of times the protein can be converted between stable states before the device is no longer reliably functional. This number is called the cyclicity, and for the application of BR in optical devices, the cyclicity has been measured to be approximately 10^6.[4,40,47,48] Recall that, for the present study, this definition translates to a net drop of the optical density at the λ_{max} from 1.0 to 0.63. Blue-shifted absorption bands, which result from denatured protein, are assumed to not contribute to the measurable absorptivity at the λ_{max} wavelength. This assumption is confirmed by the difference spectra shown in Figure S1 in the Supporting Information.

The decrease in protein absorptivity as a function of laser excitation is plotted in Figure 3 for BPR, GPR, BR, and 4-keto-BR. The absorptivity is normalized to 1.0 at the start of the experiment and after 200 photocycles protein^{-1} (600 6 mJ laser pulses). Recall that the initial absorptivity of each sample was adjusted to be ∼1.0 OD at the respective λ_{max} for each

experiment. The slopes are measured for the 0−200 and 200−400 segments and the respective values are listed in Figure 3 after multiplication by 10^6. The more negative the slope, the faster the photochemical denaturation process and the less stable the protein to photoactivation.

Blue proteorhodopsin has a considerably lower cyclicity than the other three proteins in this study. It is interesting to compare this cyclicity with that observed for the blue membranes of BR, which are generated by replacing Asp-85 with other residues and are red-shifted in λ_{max}.[49,50] It is a curious feature of both the *eubacterial* and *archaeal* retinal proton pumps that the blue-shifted variants consistently have lower photochemical stabilities than the red-shifted variants (see Figure 4 and refs 49−53). It is known that in the blue membrane of BR that there is a disruption of the hexagonal lattice, and the loss of the lattice will invariably destabilize the protein.[53] Loss of stability may also reflect the increased energy of the absorbed photon in the blue-shifted species, and the fact that this energy must be dissipated by the protein as heat during the photocycle. It should not be assumed that the lower cyclicities observed here necessarily reflect a lower cyclicity in nature. These results do suggest, however, that BPR is not amenable for use in devices requiring very high cyclicities (e.g., holographic memories).[4,40,47,48]

In contrast, GPR has an excellent cyclicity that is an order of magnitude greater than BPR and comparable to 4-keto-BR (Figure 4 and Table 1). Thus, GPR can find application in a variety of photonic devices. We will explore this issue in more detail below where the advantages and disadvantages of this protein are compared to BR.

Thermal Stability. Proteins denature when exposed to thermal stress and, as the photoactive element of the proposed technologies, PRs must withstand heat generated by these devices. Bacteriorhodopsin, which has long been the paradigm of such devices, is stable above 80 °C in an aqueous state[54−57] and to 140 °C as a dry film.[58] No such investigation of PR exists to date and understanding how PR responds to heat is vital for any practical application of these proteins. A combination of DSC and thermal bleaching experiments are done to this end. Alkaline pH is investigated because PR is photochemically functional, and thus useful for devices, only when the pH is greater than 7.[15]

Thermally induced conformational changes within the protein structure, examined via DSC, reveal that both PRs must experience high temperatures before a thermal event is observed (Figure 5). For GPR, a broad transition is followed by a narrow transition that is observed at both pH 7.4 and 8.5 (Figure 5, A and B). This denaturation is sensitive to pH, as evidenced by the decreasing T_M of GPR, in a similar manner to that of native BR (Figure S3 in the Supporting Information).[59,60] This relationship is not significantly affected when thermograms are obtained under different buffering conditions and scan rates and is consistent with pH studies on the thermal stability of native BR.[59] For BPR, only a single broad thermal transition is observed at pH 7.4 (Figure 5A) and at pH 8.5 regardless of scan rate (data not shown).

The T_Ms of BPR and GPR are sensitive to the scan rate, a characteristic feature of kinetically stabilized proteins that precludes the determination of equilibrium thermodynamic parameters from this DSC data. The E_{APP}, however, can be determined from the dependence of the T_M on the scan rate.[61] At both pH 7.4 and 8.5, preliminary determinations of the E_{APP} values are approximately 400 kJ mol^{-1} for BPR and 700 kJ mol^{-1} for the narrow transition of GPR. Recall that both PRs are detergent solubilized and BR is in the native purple membrane lipid environment. It is thusly notable that these E_{APP} determinations are of the same magnitude as those for opsin, rhodopsin, and BR in their native membranes.[42,62]

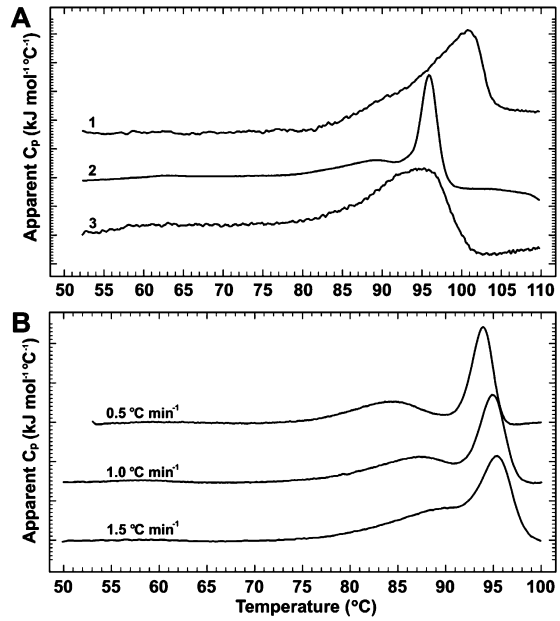


Figure 5. (A) DSC thermograms for (1) native BR, (2) OG-solubilized GPR, and (3) OG-solubilized BPR in 50 mM HEPES (pH 7.4) at a scan rate of 1.0 K min^{-1}. (B) DSC thermograms for 1 mg mL^{-1} GPR at scan rates of 0.5, 1.0, and 1.5 K min^{-1} in 50 mM TAPS at pH 8.5.

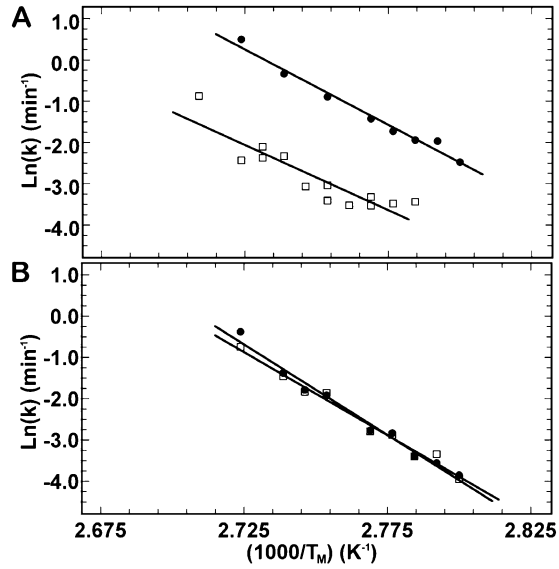


Figure 6. Activation energy for the thermal bleaching of (A) GPR and (B) BPR in 50 mM HEPES (pH 7.4) (open squares) or 50 mM TAPS (pH 8.5) (closed circles).

The activation energies for the thermal bleaching of BPR and GPR are determined from an Arrhenius analysis (Figure 6, A and B) of kinetic data obtained at multiple temperatures (Figure S4 in the Supporting Information). These data were collected between 84 and 94 °C and yield similar energies at both pH 7.4 and 8.5 (Table 2). These energies are similar to the total enthalpies observed in DSC thermograms of BPR, but not for GPR. This result indicates that BPR undergoes a one-step denaturation that involves the thermal bleaching of the protein. The denaturation of GPR is more complex, as exemplified by the dual thermal transitions observed in DSC thermograms, and

TABLE 2: Energy (kJ mol^{-1}) Required for the Thermal Denaturation of Proteorhodopsin

	pH	thermal bleaching[a]	DSC[b]
BPR	7.4	340	310 ± 40
	8.5	360	360 ± 20
GPR	7.4	300	520 ± 10
	8.5	340	440 ± 20

[a] Values represent a single set of experiments for BPR and an average of two sets of experimental data for GPR. All thermal bleaching experiments were conducted between 84 and 94 °C. [b] Values represent the total integrated heat, or enthalpy (ΔH), for DSC thermograms.

TABLE 3: Thermal Denaturation Values of Native and Mutant Bacteriorhodopsin in Distilled Water, Collected at a Scan Rate of 1.5 K min^{-1} unless Noted Otherwise

	T_M (°C)
native	99
E9Q[a]	95
R82H	100
D85N	94
D85T[b]	94
T90A	90
D96N	96
K159Q	94
Y185F	91
E194Q/E204Q[a]	94
E9Q/E74Q/E194Q/E204Q[a]	90

[a] Reference 87. [b] Thermogram was collected in 10 mM phosphate (pH 7.0) at a scan rate of 1.0 K min^{-1}.[88]

thermal bleaching occurs during the lower thermal transition. Bleaching temperatures for GPR are confirmed by brief exposure of the protein to elevated temperatures (Figure S5 in the Supporting Information).

Potential for Biotechnology. Both PR molecules exhibit a high degree of photochemical and thermal stability and are good candidates for biotechnological applications; however, their unique properties may favor application in different types of devices (Table 1). The T_Ms of both PR molecules are also comparable to those of BR mutants in the native *Halobacterium salinarum* membrane (Table 3). This comparison is of interest because, like the 4-keto-BR analogue, many BR mutants are under investigation for their application in devices.[4,63] Much remains to be understood, however, before the photochemical properties of PR can be successfully harnessed for such applications. For example, although the photocycle of BPR is reported to be an order of magnitude slower than that of native BR,[30] no detailed studies of the BPR photocycle exist. Furthermore, the conventional preparation method for studying the photophysical properties of PR is significantly different than that of BR. These discrepancies (e.g., lipid environment) make the direct comparison of BR and PR difficult and may augment complexity (e.g., photocycle kinetics). Nonetheless, the BR and PR photocycles remain strikingly similar and the potential of PR for device application is currently boundless. The ubiquitous prevalence of PR variants in marine microorganisms furthers this potential as a naturally occurring biomaterial.[28,29,64,65]

Biological Significance of These Studies. More than 4000 putative BPR and GPR genes have been identified from various oceans and seas throughout the world.[14,28,66−70] While the functional role of GPR is accepted as a light-transducing proton pump (e.g., BR),[14,19,31,70,71] the role of BPR is less obvious and remains a subject of debate. The existing literature assigns BPR as either a light-transducing proton pump[65,72,73] or a sensory pigment,[70,74] while other reports examine the relative merits of these assignments.[31,67,75,76] Functional assignments are based on a combination of experimental data collected on PR in *E. coli* phospholipids[14,28,69,70] and theoretical homology models.[31,77]

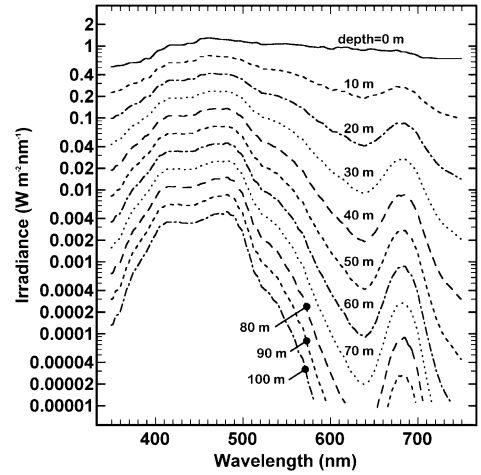


Figure 7. Irradiance of solar energy in clear, open ocean water as a function of depth. The figure is based on the data presented in ref 80.

The cyclicity and thermal data presented in this work, although collected in a detergent environment, further demonstrate the differences between BPR and GPR.[78,79] The reduced photochemical stability of BPR best exemplifies this difference and might be viewed to support those proposals that BPR is a regulatory or sensory protein.[31,70] Recall, however, that this strain of BPR (HOT75m4) is from an organism that inhabits the lower region of the euphotic zone where light is less intense (Figure 7).[14,80,81] In this region, the solar irradiance is only 0.013 of that available at the ocean surface (Figure 7). Thus, the cyclicity of BPR could be 78 times less than that of GPR and provide comparable functional longevity. The fact that the observed cyclicity of BPR is only ∼6 times lower than GPR indicates that the lower cyclicity will have no biological impact. The decreased photochemical stability of BPR is unlikely a result of a weakened structure, as evidenced by the high T_M in DSC thermograms (Figure 5), but simply the fact that natural selection creates adequate rather than superlative funcionality. We conclude that our observations are consistent with both BPR and GPR serving as proton pumps.

Comments and Conclusions

Based on the high level of thermal stability exhibited by GPR molecules, the OG-solubilized form of GPR is suitable for application in bioelectronics. There may also be applications where BPR can be used, as many devices do not require cyclicities above 10^3.[4,82] Most significant is the ability of the solubilized PR variants, described in this work, to maintain spectral activity for several years at the operational temperature of most electronic devices (i.e., ≤45 °C). Conversely, detergent solubilization of native BR results in deleterious changes to the structure and function of the protein[83,84] and illustrates a significant advantage of PR over BR. Furthermore, these data are the first to demonstrate the ability of PR to maintain spectral activity at relatively high temperature. Thus, the detergent-solubilized PR molecules are not only spectrally similar to native BR, but the green variant is shown to be a competitive alternative to BR for biophotonic devices.

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Supporting Information Available: Supporting material for this article includes (1) experimental details of measuring the photochemical stability, (2) a figure showing the difference spectra of the photochemical degradation products, (3) a figure showing the protein absorptivity versus laser excitation, (4) a figure of the Mathscriptor program to calculate and plot photochemical denaturation, (5) a figure describing the pH dependence of the GPR T_M, (6) a figure showing representative thermal bleaching data of GPR, (7) a figure showing the temperature-dependent change in the Active Population of GPR, and (8) references relevant to the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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