Photochromic Bacteriorhodopsin Mutant with High Holographic Efficiency and Enhanced Stability via a Putative Self-Repair Mechanism

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ABSTRACT: The Q photoproduct of bacteriorhodopsin (BR) is the basis of several biophotonic technologies that employ BR as the photactive element. Several blue BR (bBR) mutants, generated by using directed evolution, were investigated with respect to the photochemical formation of the Q state. We report here a new bBR mutant, D85E/D96Q, which is capable of efficiently converting the entire sample to and from the Q photoproduct. At pH 8.5, where Q formation is optimal, the Q photoproduct requires 65 kJ mol⁻¹ of amber light irradiation (590 nm) for formation and 5 kJ mol⁻¹ of blue light (450 nm) for reversion, respectively. The melting temperature of the resting state and Q photoproduct, measured via differential scanning calorimetry, is observed at 100 °C and 89 °C at pH 8.5 or 91 °C and 82 °C at pH 9.5, respectively. We hypothesize that the protein stability of D85E/D96Q compared to other blue mutants is associated with a rapid equilibrium between the blue form E85(H) and the purple form E85(−) of the protein, the latter providing enhanced structural stability. Additionally, the protein is shown to be stable and functional when suspended in an acrylamide matrix at alkaline pH. Real-time photoconversion to and from the Q state is also demonstrated with the immobilized protein. Finally, the holographic efficiency of an ideal thin film using the Q state of D85E/D96Q is calculated to be 16.7%, which is significantly better than that provided by native BR (6–8%) and presents the highest efficiency of any BR mutant to date.

KEYWORDS: blue bacteriorhodopsin, Q-state, stability, bionanotechnology, electro-optical materials, directed evolution

Bacteriorhodopsin (BR) is the light-harvesting protein expressed by the salt marsh archaeon Halobacterium salinarum, when the environment lacks sufficient free oxygen to permit oxidative phosphorylation as a source of energy.¹,² This transmembrane protein contains a covalently linked chromophore, all-trans retinal, and is arranged in trimers within a semicrystalline lattice.³–⁵ The ensemble is referred to as the purple membrane. Following the absorption of light by retinal, the light-adapted form of the protein pumps a proton across the cell membrane, thereby creating a pH gradient that then drives ATPase to synthesize ATP.²,⁶ Proton pumping is achieved by a photocycle that returns to the resting state (bR) in roughly 10 ms at ambient temperature.⁷–⁹ While chemical and genetic modifications of BR have been used to extend the lifetime of various intermediates in the main photocycle,¹⁰–¹² these methods have only recently produced highly stable intermediates for use of the protein in long-term data storage¹³–¹⁵ and associative processors.¹⁰,¹⁶–¹⁹ The ability to efficiently convert this photochromic protein from BR (λmax = 570 nm) to an inactive state, known as the Q photoproduct (λmax = 390 nm), is of interest for the successful implementation of BR into these optical devices.

The Q state is a photoproduct of the BR branched photocycle and remains stable for many years at ambient temperature.²⁰,²¹ The chromophore in Q is in a 9-cis configuration, which makes it an untenable candidate for binding to Lys-216 due to steric interactions with various binding site residues.¹⁸,²⁰,²¹ Bacteriorhodopsin does not form Q during the native photocycle, however, and requires the sequential absorption of a green photon to photoexcite the bR state and a red photon during the population of the O state (λmax = 610 nm) in order to produce Q (Figure 1).²¹ Photochemical formation of Q occurs with a lower quantum efficiency than the initial photoexcitation event (ϕ ≤ 0.65),⁷ as measured for a deionized form of the native protein. Hence, BR must be genetically modified to efficiently produce Q before the photoproduct can be utilized in protein-based technologies.¹³,¹⁵–¹⁷,²²

Blue BR (bBR) membranes contain modified proteins that exhibit an O-like resting state (bRB; λmax ~ 603 nm) and are
produced by either mutagenesis or low pH-induced protonation of the Asp-85 residue. The bBR form of the protein is a bistable photochrome that is long known to efficiently produce the pink membrane ($\lambda_{\text{max}} \sim 490$ nm), a 9-cis precursor to the Q photoproduct. Chemical bBR membranes are generated by acidification with a strong acid or deionization of the bulk protein; however, such chemical modification affects the entire protein structure and can significantly destabilize the protein conformation. The phototransformations of these bBR membranes to the Q photoproduct are also characterized by poor quantum efficiencies ($q_q \sim 2 \times 10^{-4}$ to $7 \times 10^{-4}$). Conversely, mutual manipulation of BR results in a more localized perturbation of the protein structure and provides a more advantageous method for producing stable bBR membranes. Novel methods in genetic engineering, including directed evolution, allow the photophysical properties of BR to be tailored for specific applications. Directed evolution was used to enhance the ability of BR to form the tailored for evolution, allow the photophysical properties of BR to be

We note that the letter Q is used in several contexts throughout this paper and define them here for clarity: Q represents the Q photoproduct of the branched BR photocycle, $Q_{\text{total}}$ represents a quality score used to rank BR mutants created during directed evolution, and Q in D85E/D96Q represents the amino acid glutamic acid.

### METHODS AND MATERIALS

#### Chemicals and Buffers

All chemicals were purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO). Buffers used for pH investigations were 50 mM phosphate for pH 6.5 to 7.5, 50 mM tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) for pH 8.0 and 8.5, 50 mM glycine for pH 9.0 and 9.5, or 50 mM 3-(cyclohexylamino)propanesulfonic acid (CAPS) for pH 10.0 and 10.5.

#### Strain Generation, Library Construction, and Protein Preparation

The methods for strain generation, construction of the mutant library, and the large-scale preparation and purification of bBR mutants are described in the Supporting Information. All experiments used only the high-density form of bBR proteins (see the Supporting Information methods and Figures S1 and S2), unless noted otherwise.

#### Calculation of the D85E/D96Q Molar Extinction Coefficient

The molar extinction coefficient ($\epsilon$) of the bRB and Q states of bBR mutant D85E/D96Q was experimentally determined at alkaline pH. This characterization was necessary for experiments that were conducted at pH 8.5, 9.5, and pH 10.5 to correct for the blue-shifted $\lambda_{\text{max}}$ at alkaline pH (Figure S3). Buffer exchange was done three times by using ultracentrifugation (50,000 rpm for 20 min at 4 °C), with resuspension of the protein in the appropriate buffer and equilibration for 30 min at ambient temperature after each exchange. The $\epsilon$ was experimentally determined by manipulation of the Beer-Lambert law using spectra of the bRB and Q states at each pH. Light-adaptation of either the bRB or Q states was done using either white light (300 W) for 1 h at ambient temperature for the bRB state or red light (>640 nm; 100 mW cm$^{-2}$) overnight at 30 °C induce the Q photoprotect.

#### Preparation of the Q Photoprotuct

A 1 mg mL$^{-1}$ sample of bBR membrane was prepared at an alkaline pH in a clear microcentrifuge tube (Thermo Fisher Scientific, Inc.). The sample was then placed under red LED irradiation (100 mW cm$^{-2}$; >640 nm) overnight at 30 °C. Upon completion, an aliquot was taken for analysis before the sample was wrapped in foil and stored at 4 °C until use.

#### Differential Scanning Calorimetry

All calorimetric experiments were done with 1 mg mL$^{-1}$ protein using a Microcal VP-DSC (Amherst, MA). Dialysis of all samples versus three one-liter volumes of the appropriate buffer was determined to generate the most reproducible data. The Q photoprotect was produced as described above, and because it is light sensitive, all manipulation was conducted under dim red light.

Kinetic DSC experiments were conducted at various scanning rates (30 to 90 K h$^{-1}$) based on experimental design. The melting temperature ($T_m$) of transitions within DSC thermograms were extracted and fit to the kinetic equation

$$
\ln \left( \frac{v}{T_m} \right) = \text{const} - \frac{E_{\text{app}}}{RT_m}
$$

where $v$ is the scan rate (K min$^{-1}$), $T_m$ is the melting temperature (K) from DSC thermograms, $E_{\text{app}}$ is the apparent energy of thermal denaturation (kJ mol$^{-1}$), and $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$).

Figure 1. Main and branched photocycles of native BR. The kinetic lifetimes of the BR photostates, with the respective absorption maxima (in nanometers) in parentheses, are from refs 20 and 67.

![Figure 1. Main and branched photocycles of native BR.](image-url)
Determining the Activation Energy for Q Formation and Reversion. Specialized equipment was designed to measure the ability of the D85E/D96Q mutant to switch between the \( b_{RB} \) and \( Q \) photostates at alkaline pH. The experimental setup suspends a set of four amber Luxeon III Lambertian LEDs (590 nm, 0.3 mW cm\(^{-2}\)) or three UV mcd LEDs (390 nm, 0.02 mW cm\(^{-2}\)) above the sample holder of a Cary 50 UV-visible spectrophotometer with a peltier sample cell. The experiment began by collecting an initial set of spectra of D85E/D96Q in either the \( b_{RB} \) or \( Q \) state (OD \( \sim \) 0.1) at \( t = 0 \) min before turning on the LED device and monitoring light-induced changes by collecting spectra in three stages (1 min intervals to 10 min; 5 min intervals to 50 min; 30 min intervals to 300 min). The kinetic data were then fit to a first order exponential decay rate

\[
A_t = A_\infty + (A_0 - A_\infty)e^{-kt}
\]

where \( A_t \) is the absorbance at time \( t \), \( A_\infty \) is the absorbance at time infinity, \( A_0 \) is the absorbance at time zero, \( k \) is the decay or rise rate (min\(^{-1}\)), and \( t \) is the time (min). Experiments were done in triplicate at temperatures between 36 and 50 °C and then fit to an Arrhenius plot to approximate the activation energy for the transition.

Preparation of Hydrated Polymer/bBR Cuvettes. Bacteriorhodopsin was immobilized within a 5% polyacrylamide matrix that was buffered with 50 mM TAPS (pH 8.5). The optical density (OD) of each sample was approximately 1 at the absorption maximum of the \( b_{RB} \) state. To prepare the polymer-based cuvettes, the BR solution was first sonicated on ice for 60 seconds using 10-second intervals, to ensure homogeneity, and was then filtered using 5 \( \mu \)m filter paper into a sterile falcon tube. Ammonium persulfate (400 \( \mu \)L) was added, and the solution was degassed for 30 minutes. The protein solution was transferred equally into either 4.0 mL or 1.5 mL methacrylate cuvettes (Plastibrand Cuvettes, Fisher Scientific, Inc.) that were optically transparent on all sides. Next, 0.5% (v v\(^{-1}\)) tetramethylethylenediamine (TEMED; Fisher Bioreagents, electrophoresis grade, assay 97%) was added to each cuvette, mixed well, and allowed to polymerize at ambient temperature. A 15% (w v\(^{-1}\)) polyvinyl alcohol (PVA; Aldrich, 99+% hydrolyzed, avg. MW 89-98,000) solution, which was degassed and buffered with TAPS, was applied to fill the remaining headspace within the cuvette. The cap was sealed using a chemically inert adhesive and was wrapped with Parafilm to prevent condensation within the cuvette.

Real-Time Q Formation Experiments. Specialized equipment was designed to measure the ability of the D85E/D96Q mutant to switch between the \( b_{RB} \) and \( Q \) photostates. The experimental setup is comprised of a Cary 50 UV-visible spectrophotometer, a modified sample cell holder with holes for LEDs on either side of the cuvette, one royal blue (0.3 mW cm\(^{-2}\); 450 nm) and one amber (0.2 mW cm\(^{-2}\); 590 nm) Luxeon III LED, and a 1014 Phidget (Alberta, Canada) board. The on/off times of the LEDs were computer controlled via a...
indicate an improvement in throughout the six rounds of mutagenesis, eleven mutants with D85N and D85E/D96Q (Figure 2). The D85E/D96Q mutant to enhance the native protein. Six rounds of directed evolution were performed and thus is produced in minimal amounts within the formation of the native protein function as volume transmission holograms due to the small size of the protein (50 nm diameter) relative to the activating di traction efficiency of an absorption grating is 3.7%, mixture absorption and refraction holograms facilitated by the photophysical properties of BR can have diffraction efficiencies that approach 100%; the equations used for the Kramers-Kronig relationship and Kogelnik approximation are provided in the Supporting Information and have been previously used to describe the br→M photoreaction of BR, in addition to the brQ and Q state analysis of D85E/D96Q, we performed the calculation for the br/Q photochromic pair of the high Q-forming mutant, V49A, using solution spectra collected at pH 8.5. All samples were prepared, and all spectra were collected as described above. 

RESULTS AND DISCUSSION

Directed Evolution of BR. Many of the current biophotonic technologies that utilize BR as a photochromic material use the transient M photostate (see ref 49 and references therein). The Q photoproduct can substitute for M in many of these applications with the added advantage of being a stable photoproduct with a more blue-shifted λmax that yields better separation of the photchromic pair. However, formation of the Q photoproduct is not beneficial to H. salinarum and thus is produced in minimal amounts within the native protein. Six rounds of directed evolution were performed to enhance the Q formation properties of BR, as a quality score (Qtotal) was experimentally measured to assess the ability of each mutant to form and revert from the Q photoproduct. Native BR exhibits a Qtotal of ~15 (pH 7), and higher values indicate an improvement in br→Q and/or Q→br photoreactions, with the highest Qtotal score being 977 for a quadruple mutant of BR. Of the 1604 unique mutants characterized throughout the six rounds of mutagenesis, eleven mutants with red-shifted absorption maxima (λmax = 585–610 nm) were identified (Figure 2). These bBR mutants all contain either one or two mutations to the native protein sequence. Attempts to introduce additional mutations to double bBR mutants resulted in either no or significantly low yields of protein.

Two bBR mutants had Qtotal values greater than 850: V49A/D85N and D85E/D96Q (Figure 2). The D85E/D96Q mutant exhibited the greatest Q formation ability of any bBR mutant and expressed well (2–4 mg per liter of culture) under preparation methods described in the Supporting Information. While the conditions particular to the formation of Q are characterized below, we note that D85E/D96Q is capable of converting the entire sample (>99% efficiency) to the Q state at slightly alkaline pH (Figure 3, circles). We also note that the enhanced sensitivity and holographic properties of V49A/D85N are already known, but this mutant is reported to have <1% conversion to the Q state. Conversely, we find that V49A/D85N can produce Q with 30–60% efficiency, depending on the pH, when immobilized in a polyacrylamide matrix (Figure 3, inverted triangles). However, the V49A/D85N mutant is not capable of full conversion to a stable Q photoproduct, and the subsequent sections will thus characterize Q using D85E/D96Q.

Spectral Titration of D85E/D96Q. Interactions within the active site of BR mutants are conventionally characterized via the purple-to-blue transition. This transition represents the physical conversion of the protein pigmentation (i.e., λmax), as is defined by the protonation state of the D85 counterion. The native protein exhibits a pKa of ~3 for this shift, where mutational bBR membranes exhibit a blue pigmentation at neutral pH and undergo a blue-to-purple transition with a pKa > 7.6,54,55

Titration of D85E/D96Q reveals a pKa at approximately pH 9.7 (Figure S3). This value is close to the value reported for the D85E mutant (9.4) and also represents the titration of the Schiff base counterion at position 85. Perturbation of the active site geometry is well documented to control the pKa of the Schiff base and thus the λmax of the protein.26,27 Nevertheless, we find that titration of D85E/D96Q caused an analogous blue-shift of the λmax to that of the D85E mutant, where both mutants absorb around 530 nm at pH 11. This blue-shifted species, which is the ‘purple’ form of D85E/D96Q, forms above the pKa and indicates the formation and stabilization of a deprotonated counterion residue E85(-). Moreover, pH values below the pKa stabilize the protonated form of E85, E85(H), which is the red-shifted species that is consistent with the acid blue membrane.28,55 Hence, the observed broad absorbance of D85E/D96Q likely represents a dynamic equilibrium between E85(H) and E85(-), which reflects differences in both protein stability and Q formation and reversion (see below). The D85E/D96Q mutant irreversibly denatured at highly alkaline pH (i.e., >11), as evidenced by a yellow hue of the sample (λmax ≈ 360 nm) and inability to revert the protein with the addition of acid. Alkaline
denaturation of D85E/D96Q is consistent with earlier reports of other mutant bBR membranes.31,56

**Thermal Stability of D85E/D96Q.** The thermal stability of native BR is conventionally quantified by measuring two thermal transitions, which are centered at 80 °C and 100 °C in DSC thermograms.57 In native BR, these transitions represent a reversible thermal relaxation of the protein and irreversible denaturation, respectively.58−60 The irreversible thermal transition is observed to be within 7 °C of native BR for all tested bBR mutants (Figure S4A), indicating that there is minimal perturbation of the protein structure. These Tm data can be fit to eq 1 by collecting thermograms with varied heating rates, which estimates the apparent energy (EApp) required for thermal denaturation of the protein. This method removes the scan rate dependence of the Tm because BR is a kinetically stabilized protein,60 and is a better metric of stability than a single Tm value. The EApp of both D85E and D85E/D96Q are comparably stable to native BR, where both D85N and V49A/D85N are not (Figures S4, B and C).

We hypothesize that the native-like stability of D85E and D85E/D96Q results, at least in part, via a self-repair mechanism that is related to the protonation state of the counterion residue (see above). While this mechanism remains to be fully understood, it is clear that D85E and D85E/D96Q are more stable than chemically modified bBR membranes34,61 and other bBR mutants (Figure S4 and ref 62). This observation supports a role for the counterion residue being transiently charged versus completely neutral, which is the case for the D85N mutants. We cannot say whether this stability results from a rearrangement of the hydrogen-bonded network within BR or from proton transfer to a nearby acceptor at this time; however, the key is likely a fast equilibrium of the photochemically active blue form E85(H) with a highly stable purple form E85(−) that is accessed on a millisecond time scale to repair any thermal pre-denaturation of the blue form. The relative amount of purple to blue form is controlled via pH (Figure 3), but this protein appears to be very stable in the range 6 < pH < 10, which suggests that the purple form need not be dominant to provide the desired thermal and photochemical stability.

**Optimal Q Formation Occurs at Alkaline pH.** The active site of native BR is not amenable to the formation of the 9-cis retinal configuration.28 Under sufficient red-light illumination, however, D85E/D96Q produces significant amounts of the Q photoprocess at alkaline pH. Figure 3, in combination with Figure S3, illustrates the importance of the protonation state of the residues that direct Q formation within the active site of BR. Alkaline pH, especially pH 8.5 (see below), is optimal for hydrolyzing the 9-cis retinal within the D85E/D96Q active site. Water is essential for the hydrolysis event during the P→Q transition, and, if the protein is dehydrated, formation of the P state is favored with little or no Q production.20

Formation of Q coincides with the spectral shift of the bRb λmax at alkaline pH. The spectral shift of the D85E/D96Q λmax spans approximately 80 nm between pH 8.5 and 10.5 and determining the ε for these experimental conditions was necessary to characterize the Q state. Hence, the ε was established at three pH values (8.5, 9.5, and 10.5) to properly investigate the photophysical properties of Q formation (Table 1). We note that the ε values determined for the D85E/D96Q Q photoprocess at alkaline pH differ from the published value, which use native BR, of 33,000 M⁻¹ cm⁻¹.20 This difference not only is most likely from alterations within the binding site of the D85E/D96Q mutant but also may result from the varied hydration, which is vital for forming the Q photoprocess, in the cited work.

Real-time spectral analysis of Q formation at alkaline pH was conducted under continuous illumination with amber light (Figure 4). These data show the conversion of the O-like bRB

![Figure 4.](image)

**Table 1. Summary of the Spectral Properties of bBR Mutant D85E/D96Q.**

<table>
<thead>
<tr>
<th>state</th>
<th>pH</th>
<th>λmax (nm)</th>
<th>ε (M⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>resting bRB</td>
<td>water</td>
<td>608</td>
<td>43,000 ± 2,000</td>
</tr>
<tr>
<td>8.5</td>
<td>608</td>
<td>34,000 ± 550</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>565</td>
<td>28,500 ± 1,900</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>530</td>
<td>27,000 ± 1,200</td>
<td></td>
</tr>
<tr>
<td>Q photoprocess</td>
<td>8.5</td>
<td>390</td>
<td>40,000 ± 650</td>
</tr>
<tr>
<td>9.5</td>
<td>385</td>
<td>40,000 ± 700</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>385</td>
<td>37,000 ± 1,100</td>
<td></td>
</tr>
</tbody>
</table>

Hydration state to a blue-shifted photoprocess that is centered at ~390 nm. To confirm that the blue-shifted absorbance is the Q state, and not a denatured form of the protein, the sample was subjected to continuous blue light irradiation.63 This illumination converts the Q photoprocess back to the O-like bRB state with a rate that is influenced by the solution pH and temperature (data not shown). The latter dependence allows for determination of the activation energy barrier of thermal denaturation by Arrhenius treatment of the kinetic data (Figure S5). Energies were determined at pH 8.5 and 9.5 because these conditions are the most favorable for Q formation (Figure 3). Amber (590 nm) LEDs, rather than red (>640 nm), were used for better coupling with the protein at both pH values. For the bRB→Q and Q→bRB photoconversions, these values are 65 kJ mol⁻¹ and 5 kJ mol⁻¹ for pH 8.5 and 80 kJ mol⁻¹ and 40 kJ mol⁻¹ for pH 9.5, respectively. Activation energies were not...
investigated at pH 10.5 because the protein often denatured during the course of the experiment.

The thermal denaturation of native BR is sensitive to alkaline pH.24 Because formation of Q required such an environment (Figure 3), understanding how alkaline pH affected the thermal stability of D85E/D96Q was essential before any practical application of the protein. We find that the irreversible $T_{m}$ of the bRB state (Figure S6A) and Q photoproduct (Figure S6B) decrease with increasing pH. This sensitivity is similar to that of native BR.24 Optimal stability of D85E/D96Q was observed at pH 8.5, in which the thermogram in the pH 9.5, respectively. These data show that D85E/D96Q is the 9-

The left inset image of Figure 5 shows hydrated suspensions (Figure S7). For the bRB and Q conformations, these values are 770 and 520 kJ mol$^{-1}$ for pH 8.5 and 100 and 150 kJ mol$^{-1}$ for pH 9.5, respectively. These data show that D85E/D96Q is significantly destabilized above pH 8.5, despite an increased energy barrier for the Q$\rightarrow$bRB from 5 to 40 kJ mol$^{-1}$. Hence, optimal stability of and photoconversion after illumination with (>640 nm; ∼100 mW cm$^{-2}$) light for complete conversion after 16 h.

**Table 2. Summary of the Physical Properties of bBR Mutant D85E/D96Q at Neutral and Alkaline pH**

<table>
<thead>
<tr>
<th>photostate</th>
<th>pH</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$T_{m}$ (°C)</th>
<th>$E_{app}$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>resting state (bRB)</td>
<td>water</td>
<td>nd$^b$</td>
<td>98</td>
<td>1050</td>
</tr>
<tr>
<td>8.5</td>
<td>65</td>
<td>100</td>
<td>770</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>80</td>
<td>91</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>nd$^c$</td>
<td>78</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Q photoproduct</td>
<td>8.5</td>
<td>5</td>
<td>89</td>
<td>520</td>
</tr>
<tr>
<td>9.5</td>
<td>40</td>
<td>82</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>nd$^c$</td>
<td>65</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Activation energy for the bRB$\rightarrow$Q or Q$\rightarrow$bRB photoconversion. 
$^b$The $T_{m}$ represent values collected at 90 K h$^{-1}$. $^c$nd = not determined.

**Application in Biophotonic Devices.** Optical memories can use either dry or hydrated suspensions of the protein.10,15,18,28 Formation of the Q photoproduct requires hydrolysis of the Schiff base linkage between the protein and the 9-cis retinal.20,21 We therefore investigated whether D85E/D96Q was useful in devices by suspending the protein in a hydrated polyacrylamide matrix. Such devices have been proposed for the storage and manipulation of data and offer a simple assessment of this quality.13,15

The left inset image of Figure 5 shows hydrated suspensions with the mutant protein in either the bRB or Q photostate. Both samples were prepared at pH 8.5, which is the best pair for bRB/Q cyclicity and stability, with an OD of ∼1 at 600 nm. One sample was then illuminated with continuous red light for 16 h to ensure complete formation of the Q photoproduct. Spectral analysis of these samples detected the photochemical transition to the Q photoproduct. Optical clarity was also maintained, as evidenced by the minimal absorbance at high wavelengths (>700 nm) during the photoconversion and by no denaturation of the protein following immobilization within the polyacrylamide matrix.

Real-time cycling between the bRB and Q states was also demonstrated by subjecting the sample to pulses of amber (590 nm) and royal blue (450 nm) light (Figure 6). Illumination using the amber or royal blue LEDs resulted in a decrease or increase of the baseline at 640 nm, respectively. This wavelength is used to monitor the red-shifted component of the bRB state as the protein converted to and from the Q photoproduct. Formation of Q was confirmed by the lasting blue-shifted absorbance at ∼390 nm after the protein relaxes during the dark period that follows illumination (Figure 6B). Blue irradiation reset the baseline at 640 nm by driving the Q$\rightarrow$bRB photoreaction (Figure 6, A and C). While reversion of the protein to the bRB state initially produced an increase in the postillumination baseline, this absorbance slowly decayed to the pre-experiment baseline within several minutes. This slow decay corresponds to the formation of a long-lived, red-shifted photointermediate ($\lambda_{max} = 620$ nm), presumably an O state, that then relaxed to the bRB resting state of D85E/D96Q. The photosresponsse of the immobilized protein to variable pulses of light over the course of several continuous experiments is further demonstrated in Figure S8.

**Holographic Efficiency of Q.** The blue-shift associated with Q also has application in protein-based associative memories, which traditionally use the refractive index change of the BR$\rightarrow$M photoreaction for real-time holography.11,15 Specifically, Q has two advantages over M in such applications. First, the Q photoproduct is stable for years,20 while the longest lived M state exists for ∼750 ms in the D96N mutant.55 Second, Q is more blue-shifted than M by ∼20 nm, which increases the spectral separation of the photochromic pair. However, the efficient formation of Q has only recently been realized through significant mutagenesis of BR.22 This achievement now allows us to calculate the holographic efficiency of BR mutants using experimental data.

The holographic efficiency of D85E/D96Q was calculated from solution spectra for an ideal film with an OD of ∼S at 280 nm (OD$\text{tot} \sim 1.9$) (Figure 7A). The maximal diffraction efficiency of such a film is 16.7% at 700 nm, which is notably improved over the 6–8% observed for native BR.15,57,58 It is also improved over the 4% efficiency of V49A/D85N bBR, which uses P ($\lambda_{max} = 480$ nm) as the blue-shifted species.28 This observation means the bRB/Q states of D85E/D96Q can improve the signal-to-noise of a holographic film 2–3 times over the bBR/M pair of a native BR film and 3–4 times over the bRB/P pair of V49A/D85N.
Figure 6. Illumination of D85E/D96Q, which is suspended in a buffered (pH 8.5) polyacrylamide gel, with amber (590 nm) or royal blue (450 nm) light demonstrates how the protein responds to LED illumination. (A) Real-time changes in the red-shifted absorbance band (640 nm) of the immobilized protein. Amber illumination consists of two sets of five 15-second pulses with each pulse followed by 5 seconds of dark time. Each set of pulses is followed by one minute of dark time to allow relaxation of the protein to the bRB state if unconverted to the Q photoproduct. Two minutes of blue illumination are included to drive the Q→bRQ photoconversion and reset the A640 baseline. (B) Difference spectra for the bRQ→Q photoconversion, which are subtracted from spectra of the light-adapted protein. All spectra were collected during the long dark period following each set of five amber illuminations. The number of 15-second flashes required to produce each spectra are shown on the right side of the image. The kinetics of photoconversion are shown in the inset image for the absorption maxima and minima. (C) Data are of difference spectra for the Q→bRQ photoconversion and are subtracted from the spectra of D85E/D96Q after 385 15-second amber pulses. All spectra were collected during a 30 second dark period following each one second blue illumination. The number of flashes required to produce each spectra are denoted on the right side of the image. The kinetics of photoconversion are shown in the inset image for the absorption maxima and minima.

We next calculated the holographic efficiency for the bR/Q states of V49A, a ‘purple’ BR mutant capable of fully forming Q2, in order to compare it with that of D85E/D96Q. An ideal film with an OD280 of ~5 (OD270 of ~2.4) will yield a diffraction efficiency of 12.5% at 650 nm (Figure 7B), which improves the diffraction efficiency 1.5–2 times over the bR/M pair of a native BR. This improvement, while significant, is less than that of D85E/D96Q due to the increased spectral separation between the bRQ and Q states. While both proteins offer an improved holographic efficiency, D85E/D96Q exhibits the highest efficiency of any BR mutant explored to date.10,58,37,50,66

**COMMENTS AND CONCLUSIONS**

We report here the first bBR mutant to exhibit efficient conversion of the entire sample to and from the Q photoproduct. The thermal and photochemical stability of native BR makes it well-suited for application in biophotonic technologies, and this stability is not lost in the D85E/D96Q mutant. This mutant, in particular, also exhibits the highest conversion levels to the Q photoproduct of any bBR mutant created to date. The protein structure is partially weakened, however, under the alkaline conditions required for the formation of the Q photoproduct. Optimal stability and formation of Q is determined to occur around pH 8.5 in an aqueous environment. We hypothesize that the general stability of D85E/D96Q derives from the rapid interconversion of the protonated blue form, E85(H), and the deprotonated purple form, E85(−), of the mutant. The barrier to proton transfer is very small, and thus this equilibrium is established within seconds, if not milliseconds. Rapid equilibration between these species serves to stabilize the blue membrane form, E85(H), and is likely responsible for stabilizing the ensemble.

Suspension of D85E/D96Q in a polyacrylamide matrix, buffered at pH 8.5, does not inhibit the formation of Q under the conditions optimized for the aqueous sample. Furthermore, the Q state is the only true photoproduct of the BR photocycle and, because it is infinitely longer-lived than the M65 or P18 photostates, it is ideal for long-term storage of binary data or holographic associative processing.15 Real-time cycling of the bRQ and Q states of immobilized D85E/D96Q with amber and blue light demonstrates this feature. The increased spectral separation between the photochromic pair, bR/Q, and a high holographic efficiency of D85E/D96Q also surpasses the efficiency of all protein-based holographic media investigated to date. The coupling of this efficient photochromism with the uniquely enhanced thermal stability of D85E/D96Q dramatically improves the viability of using bBR mutants in protein-based optical computing (e.g., permanent data storage, real-time holographic data processing). Following the success of immobilizing the protein within a hydrated polymer matrix and the demonstration of repeatable photoconversion in this environment, we are now eager to investigate the application of D85E/D96Q as a photochromic material and further establish the optical and hardware requirements to harness this efficiency in various biophotonic architectures.
Figure 7. Calculated wavelength dependence of the holographic efficiency for D85E/D96Q (A) and V49A (B) BR using the Kramers-Kronig transformation and Kogelnik equations. The holographic efficiency (purple line) was calculated for 100% conversion of state 1 (bR$_Q$ or bR; thick red line) to state 2 (Q; dotted blue line) in a 0.01 cm thin film with an OD of 5 at 280 nm. A 10$^\circ$ write angle was used with a write wavelength of 532 nm. The refractive index change (shaded green line) and holographic efficiency are normalized to their maximal values. All spectra represent data collected at ambient temperature.

**ASSOCIATED CONTENT**


**REFERENCES**


