Bacteriorhodopsin D85N: Three Spectroscopic Species in Equilibrium†

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ABSTRACT: Ground-state absorbance measurements show that BR from *Halobacterium halobium* containing asparagine at residue 85 (D85N) exists as three distinct chromophoric states in equilibrium. In the pH range 6–12 the absorbance spectra of the three states are demonstrated to be similar to flash-induced spectral intermediates which comprise the latter portion of the wild-type BR photocycle. One of the states absorbs maximally at 405 nm, has a deprotonated Schiff base, and contains predominantly the 13-cis form of retinal, identifying it as a close homologue of the M intermediate in the BR photocycle. The other species possess absorbance maxima with correspondence to those of the wild-type N (570 nm) and O (615 nm) photointermediates. The retinal composition of the O-like form was found to be dominated by all-trans isomer. The pH dependence of the concentrations of the equilibrium species corresponds closely with the pH dependence of the M, N, and O photointermediates. These data support kinetic models which emphasize the role of back-reactions during the photocycle of bacteriorhodopsin. Energetic and spectral characterization of the D85N ground-state equilibrium supports its use as a model for elucidating molecular transitions comprising the latter portion of the BR photocycle.

Bacteriorhodopsin (BR)[1] is the most abundant retinal binding protein found in the salt-loving Archaebacterium *Halobacterium halobium*. This member of the seven transmembrane spanning family functions as a light-driven proton pump (Oesterhelt & Stoeckenius, 1973) to generate a membrane potential which supports energy-requiring cellular processes (Danon & Stoeckenius, 1974). The photocycle is initiated by light absorption which stimulates BR into an excited state that thermally decays through a series of optically distinct intermediates. The conversions between intermediates are coupled to proton-transfer reactions (Stoeckenius & Bogomolni, 1982; Lanyi, 1992; Oesterhelt et al., 1992; Rothschild, 1992). The number and structures of functionally relevant molecular states which occur during the BR photocycle and the chemical and physical factors which regulate the transitions between these states need to be elucidated to understand the molecular mechanism of light-mediated proton translocation in BR. Understanding of this mechanism has been advanced by kinetic modeling of optical transitions between photocycle intermediates observed for wild-type BR and variant BR proteins containing single amino acid substitutions (Varo & Lanyi, 1990a; Thorgeirsson et al., 1991; Lozier et al., 1992).

The complexity of the light-induced wild-type photocycle is evident from the variety of models invoked to describe optically observed kinetic transitions between intermediate states. Models have included five to eight photointermediates and have differed in the number and ordering of rate-limiting steps and by inclusion of branching pathways and parallel cycles (Chu Kung et al., 1975; Lozier et al., 1975; Sherman et al., 1979). It has recently been proposed that a number of the photocycle intermediates (L, M, N, and O) are nearly isoenergetic, and as a consequence, back-reactions need to be accounted for in photocycle data reduction (Chernavskii et al., 1989; Ames & Mathies, 1990; Varo et al., 1990; Varo & Lanyi, 1991a). In addition, Lozier and colleagues (1992) have proposed a near-equilibrium model to describe the transitions between the intermediates. Direct thermodynamic analyses can test and perhaps verify these models of the BR photocycle.

Electrostatic interaction between amino acid side chains and the chromophore retinal regulates the transitions between intermediate states of the functional cycle (de Groot et al., 1989; Bashford & Gerwert, 1992). The complex counterion environment of retinal is accessible to solvent (Fischer & Oesterhelt, 1979; Henderson et al., 1990; Der et al., 1991; Jonas & Ebrey, 1991) and has been proposed to include numerous ionizable amino acid residues (Eisenstein et al., 1987). A critical role for aspartates has been confirmed by mutagenic strategies (Braiman et al., 1988; Gerwert et al., 1989; Duñach et al., 1990; Rothschild et al., 1990; Needleman et al., 1991). Aspartate 85 (D85) is a key amino acid in this complex (Butt et al., 1989; Stern et al., 1989; Lin et al., 1991).

Replacement of D85 by asparagine (D85N) has dramatic consequences for the absorbance maximum, proton-pumping activity, and protein conformation. Acid-mediated protonation of aspartate 85 in the ground state of wild-type BR results in a blue chromophore ($pK_a \sim 3.0$) (Fischer & Oesterhelt, 1979; Kimura et al., 1984; Varo & Lanyi, 1989). D85N is an uncharged aspartate 85 analogue and is blue (λ_{max} 615 nm). Upon excitation by light and at neutral pH, D85N monomers arrest their photocycle after formation of two intermediates (Thorgeirsson et al., 1991) and do not translocate

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[1] Abbreviations: BR, bacteriorhodopsin; D85N, aspartate 85 to asparagine BR mutant expressed in *Halobacterium halobium*; SB, Schiff base; D85, aspartate 85; λ_{max}, wavelength at maximum absorbance; PSB, protonated Schiff base; kbp, kilo base pairs; PCR, polymerase chain reaction; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; *bop*, bacterioopsin gene; psi, pounds per square inch; PM, purple membrane; DA, dark adapted; OD, optical density; SVD, singular value decomposition; e-BR, *Escherichia coli* expressed BR, e-D85N, *E. coli* expressed D85N; ΔG, Gibbs free energy.

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protons (Mogi et al., 1988). These data indicate a functional role for aspartate 85 in the wild-type photocycle as (i) the direct counterion for the protonated Schiff base (PSB) (Lin & Mathies, 1989; Ames & Mathies, 1990; Marti et al., 1991) and (ii) the recipient of the Schiff base proton during formation of the M photocycle intermediate (Braiman et al., 1988; Gerwert et al., 1989).

The only published studies of the D85N mutation have reported characterization of protein expressed in *Escherichia coli* (Karnik et al., 1987; Shand et al., 1991). BR purified from *E. coli* can be regenerated with retinal in functional monomeric form, in the presence of exogenous lipids and detergents (Braiman et al., 1987; Miercke et al., 1991). The photocycles of wild-type and site-directed mutants of *E. coli* expressed BR (e-BR) have been shown to be perturbed in detergent (Milder et al., 1991; Thorgeirsson et al., 1991) and detergent–lipid environments (Varo & Lanyi, 1991a; Lanyi et al., 1992). Therefore, it is important to assess protein properties in the native lipid environment of halobacteria. We have transformed genes encoding both the wild-type and D85N proteins into BR-deficient strains of *H. halobium* using a halobacterial shuttle vector. Subsequent characterization of the D85N protein isolated from halobacteria has elucidated previously unrecognized aspects of the molecular role of the D85 carboxylate in the BR photocycle.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. A fragment containing the *bop* gene was isolated from wild-type *H. halobium* genomic DNA using polymerase chain reaction (PCR), cloned into the *E. coli* plasmid pUC19 (Yanisch-Perron et al., 1985), and subsequently transferred into the *E. coli*/*H. halobium* shuttle vector pUBP2 (Blaseio & Pfeifer, 1990). pUBP2 confers resistance to mevinolin, an HMG-CoA reductase inhibitor as a selectable marker. PCR was also used to isolate a DNA fragment containing the Asp 85 to Asn substituted *bop* gene from the *E. coli* expression vector pβgbop in which the mutation had previously been isolated (Shand et al., 1991). The *bop* gene along with upstream sequences found in *H. halobium* was reconstituted using the purified PCR fragment containing the mutation and cloned into pUBP2.

BR Expression and Harvest. pUBP2 containing either the wild-type *bop* gene or D85N was transformed into the BR-deficient *H. halobium* strain L33 (Wagner et al., 1983) by the method of Cline et al. (1989). In numerous transformation experiments, recombination between the pUBP2 plasmid bourne and chromosomal *bop* gene copies was never observed as assayed by Southern analysis (data not shown). Mevinolin-resistant D85N or wild-type colonies were harvested from transformation plates into 20 mL of halobacterial medium (Shand & Betlach, 1991), supplemented with 10–15 mM mevinolin, and grown aerobically to the midlogarithmic stage. These cultures were used to inoculate 11 L of halobacterial medium (pH 7.0) prewarmed to 40 °C in a stainless steel Microgen SF-116 fermentor (New Brunswick Scientific Co.) and grown according to the method of Lorber and DeLucas (1990) with aeration at 500 psi. Samples were harvested according to the methods of Oesterhelt and Stoeckenius (1974) using linear sucrose gradients (25–45%, 50% cushion). Membranes banding at the sucrose cushion interface were collected. Sucrose was replaced by 30 mM $NaPO_4$ (pH 6) by centrifugation, and the samples were retinylated overnight by addition of *all-trans*-retinal. Excess retinal was removed by two water washes, and membranes were reisolated through sucrose gradients. This material was used for all subsequent characterization and will be referred to as D85N or PM.

Dark-Adapted Absorption Spectra of BR as a Function of pH. Starting near neutral pH, spectra were obtained in ~0.5 pH unit increments at 20 °C using a Shimadzu UV-160 spectrophotometer. Protein samples were 2 mL at 0.3–0.4 OD in 0.1 M NaCl. Separate samples were used for the basic (pH 7–11.5) and acidic (pH 1–7) titrations. pH was adjusted using microliter volumes of HCl or NaOH and equilibrated, with stirring, for 3 min directly in a thermostated cuvette. pH was monitored with a gel-filled combination electrode (Sensorex No. SG900C) and a PHM85 Radiometer pH meter.

Spectral Analysis. Spectra were reduced and component species separated by singular value decomposition analysis (Golub & Reinsch, 1970; Thorgeirsson et al., 1991; Henry & Hofrichter, 1992). The spectra of the component species were determined by the following: given that $\mathbf{E}(\lambda)$ is a matrix containing the component species spectra and $\mathbf{C}$ is a matrix containing their concentrations as a function of pH, the following is obtained.

$$\mathbf{A}(\lambda,\mathrm{pH}) = \mathbf{E}(\lambda)\mathbf{C} = \mathbf{USV}' \tag{1}$$

where $\mathbf{A}$ is the $m \times n$ matrix containing the absorbance measurements at m wavelengths and n pH values. The matrix $\mathbf{U}$ contains the orthonormal basis spectra, $\mathbf{S}$ the associated eigenvalues, and $\mathbf{V}'$ the pH dependence of the basis spectra. In order to understand the transitions in $\mathbf{V}$ the pH 6–11.5 data set was fit to sums of Henderson–Hasselbalch terms of the following form:

$$\mathbf{A}(\lambda,\mathrm{pH}_i) = \sum_{j=1}^{n} \frac{h_j(\lambda)}{1 + 10^{(\mathrm{p}K_j - \mathrm{pH}_i)}} + h_0(\lambda) \tag{2}$$

where $\mathbf{A}(\lambda,\mathrm{pH}_i)$ is the spectrum at each pH_i and $h_j(\lambda)$ are the spectra changes associated with the n observed transitions. The fitting incorporated a simplex algorithm modification (Thorgeirsson et al., 1991) for multicomponent spectral analysis of pH titrations (Shrager & Hendler, 1982). With the spectra for the different forms $\mathbf{E}(\lambda)$ known, the concentrations of individual species can be calculated by finding the linear transformation b, such that

$$\mathbf{E}(\lambda)b = \mathbf{US} \tag{3}$$

This will yield the concentration of species through

$$\mathbf{C} = b\mathbf{V}' \tag{4}$$

where b is the least squares minimized solution to eq 3.

Retinal Extractions. Retinals were extracted and quantitated using the procedure of Scherrer et al. (1989) with modifications. Chromatography was performed using a single Zorbax-Sil column (4.6 × 250 mm) with 6% diethyl ether in hexane mobile phase. Molar extinction coefficients of 38 770 and 48 000 were used to quantitate the 365-nm peak area of the 13-cis and all-trans isomers, respectively (Liu & Asato, 1984).

RESULTS

pH-Induced Spectral Equilibria. The effect of pH on the ground-state absorbance characteristics of PM and D85N was investigated. At neutral pH the absorbance maxima of PM and D85N were determined to be 560 and 615 nm, respectively. The absorbance properties of PM were invariant over the pH range of 4–12, but a reversible transition between two spectral components was observed between pH 1 and 4 (Figure 1a,c). In contrast, D85N was composed of at least three distinct spectroscopic species, in equilibrium, over the entire pH range (Figure 1b,d). Criteria for equilibrium were established as (i) no difference in observed absorption

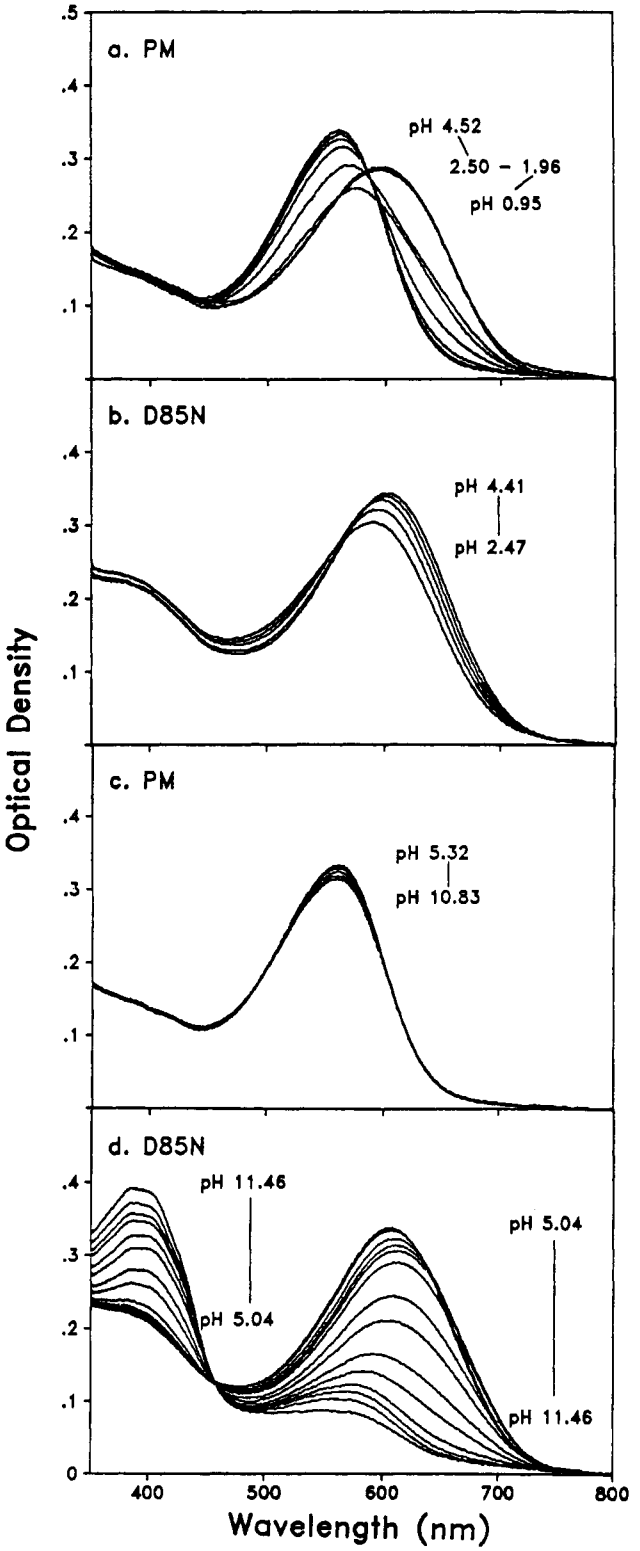

FIGURE 1: pH dependence of the dark-adapted absorption spectra of BR membranes (20 °C, 100 mM NaCl). Panels a and c contain spectra obtained for the acidic (pH 1–4.5) and basic (pH 5–11) titrations of PM. Panels b and d contain analogous spectra for D85N.

spectra when equilibration times range between 1 and 5 min after reaching final pH values and (ii) the pH titrations being completely reversible. The visible absorbance spectra were stable, and overlaid, for greater than 26 h following pH titration.

Reconstructed Spectra. Singular value decomposition (SVD) was used to identify the number of spectrally independent components required to reproduce the pH-dependent changes in the D85N absorption spectra and to characterize

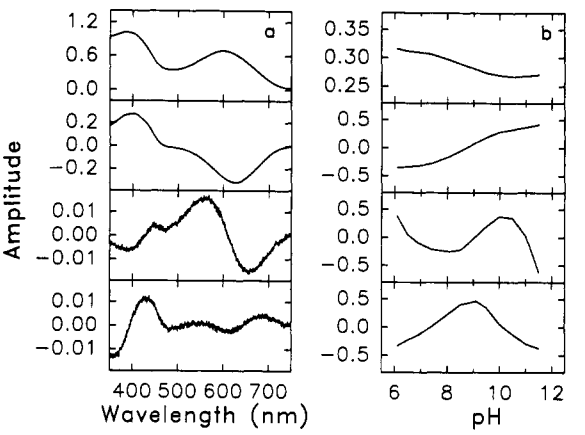

FIGURE 2: Results for the SVD analysis of the spectra of D85N. (a) The basis spectra associated with the four largest singular values shown as columns of U·S. From top to bottom: columns 1–4. (b) The corresponding pH dependence of the basis spectra. From top to bottom: columns 1–4 of V.

their optical properties. The most significant singular values associated with the basis spectra were 16.91, 5.25, 0.25, and 0.14 (Figure 2a). Higher order basis spectra (from 5 to 20) were unresolved from experimental noise. As shown in Figure 2b, the columns of V identify at least three transitions occurring in the pH range 6–12. In order to understand the pH-dependent transitions in V, a least squares fit to the Henderson–Hasselbalch relationship was performed using eq 2. Three pK values of 7.9, 9.2, and 11.5 were obtained. Figure 3a shows the calculated spectra where the transitions occur in the sequence 1 → 2 → 3 → 4. The first two pK values are associated with a transition from the blue form (λ_{max} at 615 nm) to a mixture that includes a peak at 405 nm and a species with significant absorption between 550 and 600 nm. The 11.5 pK transition is associated with a small shift in the absorbance maximum of the yellow species, consistent with the shape of the fourth basis spectrum (Figure 2a). These results demonstrate that three spectrally distinct species coexist in this pH range for D85N.

If the spectra of the three species were known, their concentrations at any pH may be calculated from eq 3. Spectra 1 and 4 in Figure 3a contain one component each, the blue and yellow forms, respectively. A spectrum for the high-pH purple species was obtained by subtracting Figure 3a spectrum 4 from spectrum 3. The difference was scaled and added to the last spectrum. The scale factor was chosen such that the absorbance at 400 nm was similar in the blue (I) and purple forms (II). The recovered spectra possess absorbance maxima at 615 (I), 570 (II), and 405 nm (III) (Figure 3b). The relative concentrations for the pH-induced D85N spectral species are displayed in Figure 4. Sums of the three individual species' concentrations give a total of 1 ± 0.02 at all pH values. The spectral changes over the pH range 6–11.5 are thus very well approximated with the spectra of Figure 3b.

Congruence of the absorbance properties for the D85N species III and the M intermediate of the purple membrane (PM) photocycle is observed. To test this correspondence, resonance Raman spectra were recorded on D85N at high pH. The 405-nm form lacked a C=N vibrational band in the 1640-cm^{-1} region, indicating that the chromophore was unprotonated. The spectra also exhibited a prominent ethylenic band at 1561 cm^{-1}, which is characteristic of the M state alone, other photointermediates containing a dominant ethylenic band near or less than 1530 cm^{-1} (Ames & Mathies, 1990). The "fingerprint region" (1100–1300 cm^{-1}) of the

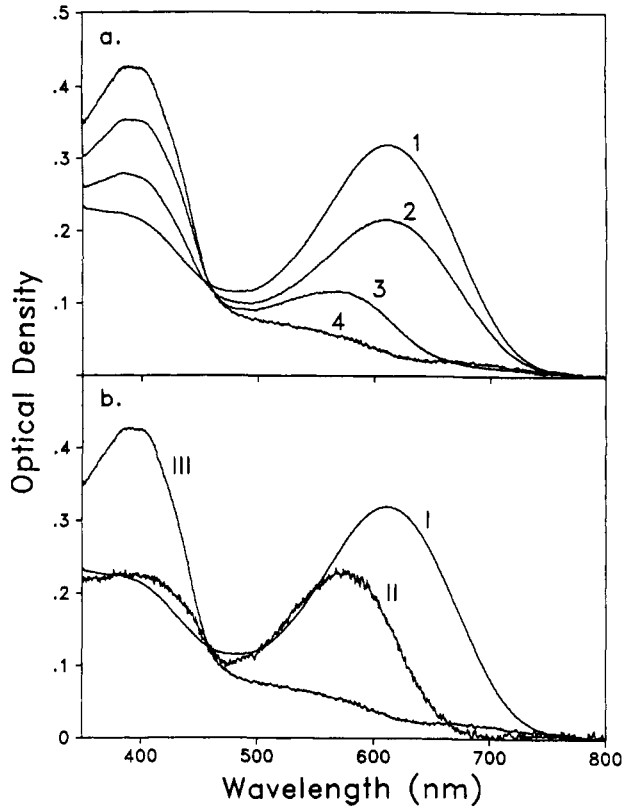

FIGURE 3: Intermediate spectra calculated for transitions in D85N. (a) Spectra representative of the Henderson–Hasselbalch analysis of V, assuming a sequential series of transitions. (b) Calculated spectra for individual species participating in the pH-induced transitions of D85N. Species II and III are components of spectra 2 and 3 and are solved by difference spectra, as described in the Results section.

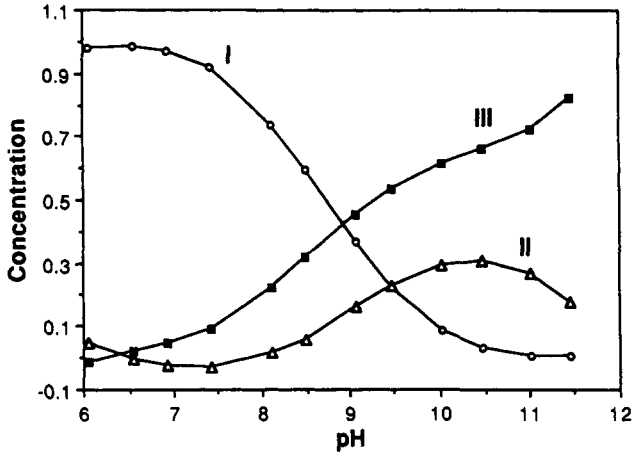

FIGURE 4: pH dependence of the concentrations of D85N species I (O), II (N), and III (M). The summed relative concentrations give a total of 1 (within 2%) at any pH.

Raman spectra showed that the isomer configuration is predominantly 13-cis (Lin and Mathies, personal communication).

To determine the isomer composition of the D85N spectral species, retinal extractions were performed at pH 6.0 and 10.5. Control experiments at neutral and high pH confirm previously reported chromophore ratios for PM in the light-adapted (100% all-trans) and dark-adapted (62% 13-cis and 38% all-trans) states (Scherrer et al., 1989). Repetitive experiments indicate less than 2% variability in our estimates of the isomer composition. At pH 6.0 D85N, which is comprised of only the O-like blue species (Figure 4), possessed a retinal composition of 61% all-trans and 39% 13-cis. The high-pH sample contained an isomer composition of 70% 13-cis and 30% all-trans. At pH 10.5 D85N is resolved as a mixture of 65% M-like yellow and 35% N-like purple species at pH 10.5 (Figure 4).

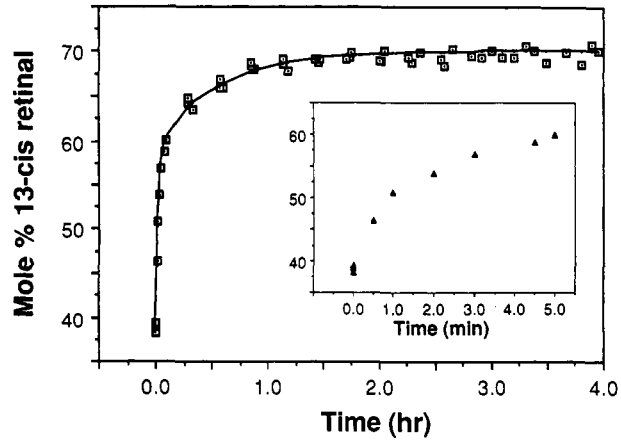

FIGURE 5: Time dependence of isomerization around the 13-carbon bond of the retinal upon the D85N low- to high-pH ground-state transition. The data are derived from six independent experiments. Extractions were performed for up to 26 h, but only the first 4 h is shown for clarity. The solid curve is the best fit to a sum of two first-order exponential reactions. The dominant phase possessed a $t_{1/2}$ of 1.1 min. The inset is an expansion of the first 5 min of the isomerization reaction.

Figure 5 shows the time dependence of isomerization around the 13-carbon bond of retinal for the D85N species upon changing the solution pH from 6.0 to 10.5. The absorbance spectra did not vary over the entire time period of the extraction experiment. Retinal isomerization around the 13-carbon bond was best described by a sum of two first-order exponential processes. The dominant phase (66% amplitude) was characterized by a 1.1-min half-time of reaction while the second smaller amplitude phase (33%) possessed a half-time of 24 min.

DISCUSSION

The intermediate states of the wild-type BR photocycle include at least three purple (BR ground state, L, and N), one yellow (M), and two blue (K and O) intermediates. In contrast, we have shown that upon photoexcitation D85N proceeds through only two photointermediates whose identities remain ambiguous (Thorgeirsson et al., 1991). Here we show that the D85N ground state exists as a pH-dependent equilibrium of at least three optically distinct species (Figure 1c,d).

The low-pH spectral transitions observed (pK's ~2.5 and ~1.5 for PM and pK ~4 for D85N) are analogous to the well-characterized acid purple to acid blue transitions in PM (Figure 1). Acid blue BR is associated with protonation of D85 (Otto et al., 1990; Subramaniam et al., 1990). The acid blue to acid purple transition (pK < 2) is mediated by anions interacting with the PSB (Fischer & Oesterhelt, 1979; Varo & Lanyi, 1989; Der et al., 1991). In 100 mM NaCl, D85N undergoes a purple (λ_{max} at 570 nm) to blue (λ_{max} at 615 nm) transition below pH 5. Asparagine at residue 85 results in a rise in both the pK of the purple to blue transition and the PSB affinity for anions in D85N.

Above pH 6 D85N can be resolved into three spectral components coexisting in a pH-dependent equilibrium. The three species possess absorbance maxima at 405 (I), 570 (II), and 615 nm (III) (Figure 3b). The correspondence between the absorbance maxima and relative extinction values with those for the O, N, and M flash-induced wild-type photocycle intermediates is striking (Lanyi, 1992; Lanyi et al., 1992; Lozier et al., 1992).

In the BR photocycle D85 is deprotonated from the ground state to L (Braiman et al., 1988). The L → M transition is linked to protonation of D85 and deprotonation of the PSB (Braiman et al., 1988; Gerwert et al., 1989). Since D85N does not undergo protonation changes at residue 85, inclusion of a K and an L analogue in the D85N equilibrium is inappropriate.

Our results correlate well with proposed mechanisms for the photocycle which suggest that D85 is protonated in O and deprotonates during the O → BR reaction (Mathies et al., 1991; Rothschild, 1992). This has been confirmed by a FTIR study that shows that D85 remains protonated past the N intermediate (Braiman et al., 1991). It is therefore likely that the ground state of D85N (Figure 3b) resembles in conformation and charge an intermediate of the latter half of the BR photocycle (i.e., M, N, and O). It is noted that the absorbance maxima for the O intermediate has been reported to range between 580 and 640 nm for PM and near 600 nm for detergent-solubilized BR (Lozier et al., 1992; Varo & Lanyi, 1991b; Thorgeirsson et al., 1991). At neutral pH the absorbance maxima of the wild-type O photointermediates, the acid blue form of PM, and the D85N species I (Figure 3b) are very similar. A correspondence of this type was first noted by Fischer and Oesterhelt (1979), who proposed that the acid blue form of PM (λ_{max} 605 nm) and the O photoproduct are identical. Subsequently Mathies and coworkers demonstrated that the Raman spectrum of acid blue PM contains an approximately equal mixture of 13-cis and all-trans chromophores (Smith & Mathies, 1985) while they concluded that the O intermediate possesses a strained all-trans retinal (Smith et al., 1983).

One of the species in equilibrium with the blue form possesses spectral characteristics of the M intermediate of the PM photocycle (i.e., λ_{max} 405 nm). Raman studies show that this species contains a deprotonated Schiff base, further supporting the analogy. The apparent pK for the formation of the M species is ~8.7 (Figure 4). e-D85N has been observed to undergo a similar blue to yellow transition with a pK = 8.8 (Duñach et al., 1990). Similar increases in pH do not cause the formation of an analogous species in BR itself, but SB deprotonation is known to occur at pH near 12 as a consequence of protein denaturation (Druckmann et al., 1982). A high-pH transition is observed in D85N as well (Figure 3a, pK ~11.5) and is assumed to be due to pH-induced protein denaturation.

Our results show that a purple form (λ_{max} 570 nm) coexists with the yellow species. This was not observed for the e-D85N material (Duñach et al., 1990). There is a close correspondence between the absorbance maximum of the N photointermediate and the high-pH purple form of D85N (Lozier et al., 1982). For PM the purple form of the chromophore is stabilized by a negatively charged counterion at the PSB. By analogy, the high-pH D85N 570-nm species can result from PSB stabilization by anions or an alternate negatively charged residue in the protein (Rothschild et al., 1990; Balashov et al., 1991; Needleman et al., 1991). BR photocycle measurements have shown that the kinetics of the transitions between the M, N, O, and BR intermediates are pH dependent. Near neutral pH the O intermediate is observed. At high pH little or no O is formed, and the N and M intermediates are seen to accumulate (Hanamoto et al., 1984; Otto et al., 1990; Varo et al., 1990; Varo & Lanyi, 1990b; Chizhov et al., 1992).

The Gibbs free energy for formation of the D85N equilibrium species may be determined from the concentrations shown in Figure 4. Employing the O-like analogue as a reference state, ΔG values of 2.3 ± 0.6 and 1.8 ± 0.3 kcal/mol were calculated for the N- and M-like forms, respectively. Only the O-like species is significantly populated at pH 7.0, and its pH dependency mimics that observed for the O intermediate in the wild-type photocycle. Consistent with recent near-equilibrium modeling of photocycle transitions, these states are observed to be nearly isoenergetic in D85N.

The absorbance properties of the species which comprise the pH-dependent D85N equilibria are essentially the same as those which result from the formation of the M, N, and O intermediates of the BR photocycle. Retinal extractions of the dark-adapted D85N show there are mixtures of 13-*cis*- and *all-trans*-retinal, consistent with dark adaptation in PM. The D85N protein isomerizations constrain a ground-state retinal transition from mostly all-trans in the O-like species to predominantly 13-cis in the M- and N-like forms. The spectra of the equilibrium species are a reflection of the structure and charge of the protein environment of retinal. Thus, while the optical spectra switch rapidly between the M-, N-, and O-like forms, they are insensitive to the isomer ratio of retinal and their interconversion is only weakly dependent on the retinal isomer composition.

Our interpretation of the D85N equilibrium species is consistent with current models for the role of protein isomerization reactions in the latter half of the BR photocycle. Conformational changes inherent in the M → N → O photocycle transitions are proposed to drive the 13-*cis*- to *all-trans*-retinal isomerization reaction resetting the BR cycle (Mathies et al., 1992, Rothschild 1992). Within the constraint of dark adaptation, the D85N transitions are observed to promote an analogous retinal isomerization. In 0.1 M NaCl the apparent pK_a's of the D85N transitions are found to be consistent with those assigned for the molecular interactions observed in the photocycle (Engelhard et al., 1985; Bashford & Gerwert, 1992). The M-like species is formed by deprotonation of the Schiff base. This species is in rapid equilibrium with the N-like form which can result from transfer of the D96 proton to the SB. The O-like species is formed concurrent with increasing proton activity and can be ascribed to reprotonation of D96 from bulk solvent. A p$K_a \geq 9.5$ has been assigned to D96 in the wild-type photocycle whereas we observe a pK_a of ~9.0 in the D85N transitions.

The D85N equilibrium may be perturbed in nonnative lipid environments. As observed by us and others, the spectroscopic and photokinetic properties of BR are sensitive to the lipid and detergent composition (Subramaniam et al., 1990; Milder et al., 1991; Needleman et al., 1991). Near neutral pH, the absorbance maximum of detergent-solubilized e-D85N is significantly blue shifted from the absorbance maximum in the presence of the native lipids (Marti et al., 1991; Miercke et al., 1991). The N ↔ O transitions may be very pH dependent in e-D85N, thus obscuring observation of an analogous equilibrium in previous ground-state studies. Recent visible absorbance measurements of e-D85N, at elevated pH, show that a blue-shifted chromophore is being formed (unpublished observation) and a pH-induced M-like ground-state analogue has been observed (Duñach et al., 1990; Marti et al., 1991).

In sum, the dark-adapted ground state of D85N expressed in *H. halobium* exists as an equilibrium between three optically distinct spectroscopic species, and the populations of these species are sensitive to modest changes in pH. These species show close correspondence with intermediate states intrinsic and essential to the photochemical activity of BR. The ability to alter the equilibrium between these three stable states in D85N may be useful in further biophysical characterization

of the M, N, and O intermediate conformational and electrostatic states.

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REFERENCES

Ames, J. B., & Mathies, R. A. (1990) *Biochemistry 29*, 7181–7190.

Balashov, S. P., Govindjee, R., & Ebrey, T. G. (1991) *Biophys. J. 60*, 475–490.

Bashford, D., & Gerwert, K. (1992) *J. Mol. Biol. 224*, 473–486.

Blaseio, U., & Pfeifer, F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6772–6776.

Braiman, M. S., Stern, L. J., Chao, B. H., & Khorana, H. G. (1987) *J. Biol. Chem. 262*, 9271–9276.

Braiman, M. S., Mogi, T., Stern, L. J., Khorana, H. G., & Rothschild, K. J. (1988) *Biochemistry 27*, 8516–8520.

Braiman, M. S., Bousche, O., & Rothschild, K. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2388–2392.

Butt, H. J., Fendler, K., Bamberg, E., Tittor, J., & Oesterhelt, D. (1989) *EMBO J. 8*, 1657–1663.

Chernavskii, D. S., Chizhov, I. V., Lozier, R. H., Murina, T. M., Prokhorov, A. M., & Zubov, B. V. (1989) *Photochem. Photobiol. 49*, 649–653.

Chizhov, I., Engelhard, M., Chernavskii, D. S., Zubov, B., & Hess, B. (1992) *Biophys. J. 61*, 1001–1006.

Chu Kung, M., Devault, D., Hess, B., & Oesterhelt, D. (1975) *Biophys. J. 15*, 907–911.

Cline, S. W., Lam, W. L., Charlebois, R. L., Schalkwyk, L. C., & Doolittle, W. F. (1989) *Can. J. Microbiol. 35*, 148–152.

Danon, A., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1234–1238.

de Groot, H. J. M., Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1989) *Biochemistry 28*, 3346–3353.

Der, A., Szaraz, S., Toth-Boconadi, R., Takaji, Z., Keszthelyi, L., & Stoeckenius, W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4751–4755.

Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., & Callender, R. H. (1982) *Biochemistry 21*, 4953–4959.

Duñach, M., Marti, T., Khorana, H. G., & Rothschild, K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9873–9877.

Eisenstein, L., Lin, S., & Dollinger, G. (1987) *J. Am. Chem. Soc. 109*, 6860–6862.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Fischer, U., & Oesterhelt, D. (1979) *Biophys. J. 28*, 211–230.

Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943–4947.

Golub, G. H., & Reinsch, C. (1970) *Numer. Math. 14*, 403–420.

Hanamoto, J. H., Dupuis, P., & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7083–7087.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckman, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Henry, E. R., & Hofrichter, J. (1992) *Methods Enzymol.* (in press).

Jonas, R., & Ebrey, T. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 149–153.

Karnik, S. S., Nassal, M., Doi, T., Jay, E., Sgaramella, V., & Khorana, H. G. (1987) *J. Biol. Chem. 262*, 9255–9263.

Kimura, Y., Ikegami, A., & Stoeckenius, W. (1984) *Photochem. Photobiol. 40*, 641–646.

Lanyi, J. K. (1992) *J. Bioenerg. Biomembr. 24*, 169–179.

Lanyi, J. K., Tittor, J., Varo, G., Krippahl, G., & Oesterhelt, D. (1992) *Biochim. Biophys. Acta 1099*, 102–110.

Lin, S. W., & Mathies, R. A. (1989) *Biophys. J. 56*, 653–660.

Lin, S. W., Fodor, S. P., Miercke, L. J. W., Shand, R. F., Betlach, M. C., Stroud, R. M., & Mathies, R. A. (1991) *Photochem. Photobiol. 53*, 341–346.

Liu, R. S. H., & Asato, A. E. (1984) *Tetrahedron 40*, 1931–1969.

Lorber, B., & DeLucas, L. J. (1990) *FEBS Lett. 261*, 14–18.

Lozier, R., Xie, A., Hofrichter, J., & Clore, G. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3610–3614.

Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophys. J. 15*, 955–962.

Marti, T., Rosselet, S., Otto, H., Heyn, M., & Khorana, H. G. (1991) *J. Biol. Chem. 266*, 18674–18683.

Mathies, R. A., Lin, S. W., Ames, J. B., & Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 491–518.

Miercke, L. J. W., Betlach, M. C., Mitra, A. K., Shand, R. F., Fong, S. K., & Stroud, R. M. (1991) *Biochemistry 30*, 3088–3098.

Milder, S. J., Thorgeirsson, T. E., Miercke, L. J. W., Stroud, R. M., & Kliger, D. S. (1991) *Biochemistry 30*, 1751–1761.

Mogi, T., Stern, L. J., Marti, T., Chao, B. H., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 29*, 4148–4152.

Needleman, R., Chang, M., Ni, B., Varo, G., Fornes, J., White, S., & Lanyi, J. K. (1991) *J. Biol. Chem. 266*, 11478–11484.

Oesterhelt, D., & Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2853–2857.

Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667–678.

Oesterhelt, D., Tittor, J., & Bamberg, E. (1992) *J. Bioenerg. Biomembr. 24*, 181–191.

Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018–1022.

Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147–167.

Rothschild, K. J., Braiman, M. S., He, Y., Marti, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 265*, 16985–16991.

Scherrer, P., Mathew, M. K., Sperling, W., & Stoeckenius, W. (1989) *Biochemistry 28*, 829–834.

Shand, R. F., & Betlach, M. C. (1991) *J. Bacteriol. 173*, 4692–4699.

Shand, R. F., Miercke, L. J. W., Mitra, A. K., Fong, S. K., Stroud, R. M., & Betlach, M. C. (1991) *Biochemistry 30*, 3082–3088.

Sherman, W. V., Eicke, R. R., Stafford, S. R., & Wasacz, F. M. (1979) *Photochem. Photobiol. 30*, 727–729.

Shrager, R. I., & Hendler, R. W. (1982) *Anal. Chem. 54*, 1147–1152.

Smith, S. O., & Mathies, R. A. (1985) *Biophys. J. 47*, 251–254.

Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. (1983) *Biochemistry 22*, 6141–6148.

Stern, L., Ahl, P., Marti, T., Mogi, T., Duñach, M., Berkowitz, S., Rothschild, K., & Khorana, H. G. (1989) *Biochemistry 28*, 10035–10042.

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 51*, 587–616.

Subramaniam, S., Marti, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1013–1017.

Thorgeirsson, T. E., Milder, S. J., Miercke, L. J. W., Betlach, M. C., Shand, R. F., Stroud, R. M., & Kliger, D. S. (1991) *Biochemistry 30*, 9133–9142.

Varo, G., & Lanyi, J. K. (1989) *Biophys. J. 56*, 1143–1151.

Varo, G., & Lanyi, J. K. (1990a) *Biochemistry 29*, 2241–2250.

Varo, G., & Lanyi, J. K. (1990b) *Biochemistry 29*, 6858–6865.

Varo, G., & Lanyi, J. K. (1991a) *Biochemistry 30*, 7165–7171.

Varo, G., & Lanyi, J. K. (1991b) *Biochemistry 30*, 5008–5015.

Varo, G., Duschl, A., & Lanyi, J. K. (1990) *Biochemistry 29*, 3798–3804.

Wagner, G., Oesterhelt, D., Krippahl, G., & Lanyi, J. K. (1983) *FEBS Lett. 131*, 341–345.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.