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UV–visible spectroscopy of bacteriorhodopsin mutants: Substitution of Arg-82, Asp-85, Tyr-185, and Asp-212 results in abnormal light–dark adaptation*

(purple membrane/proton transport/mutagenesis/retinal isomerization/kinetic spectroscopy)

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ABSTRACT **The light–dark adaptation reactions of a set of bacteriorhodopsin (bR) mutants that affect function and color of the chromophore were examined by using visible absorption spectroscopy. The absorbance spectra of the mutants Arg-82 → Ala (Gln), Asp-85 → Ala (Asn, Glu), Tyr-185 → Phe, and Asp-212 → Ala (Asn, Glu) were measured at different pH values during and after illumination. None of these mutants exhibited a normal dark–light adaptation, which in wild-type bR causes a red shift of the visible absorption maximum from 558 nm (dark-adapted bR) to 568 nm (light-adapted bR). Instead a reversible light reaction occurs in the Asp-85 and Asp-212 mutants from a blue form with λ_{max} near 600 nm to a pink form with λ_{max} near 480 nm. This light-induced shift explains the appearance of a reversed light adaptation previously observed for the Asp-212 mutants. In the case of the Tyr-185 and Arg-82 mutants, light causes a purple-to-blue transformation similar to the effect of lowering the pH. However, the blue forms observed in these mutants are not identical to those formed by acid titration or deionization of wild-type bR. It is suggested that in all of these mutants, the chromophore has lost the ability to undergo the normal 13-cis,15-syn to all-trans,15-anti light-driven isomerization, which occurs in native bR. Instead these mutants may have as stable forms all-trans,syn and 13-cis,anti chromophores, which are not allowed in native bR, except transiently.**

Bacteriorhodopsin (bR), the light-driven proton pump of *Halobacterium halobium*, has a retinal chromophore covalently bound to the protein through a protonated Schiff base linkage to the ε-amino group of Lys-216 (5). Illumination of bR causes a shift in the wavelength of maximum absorption (λ_{max}) from 558 nm (dark-adapted bR) to 568 nm (light-adapted bR). The light-adapted bR contains an all-*trans*, C=N *anti* retinal configuration. Upon extended incubation in the dark, this form thermally converts into a mixed species with an approximately equal amount of all-trans,C=N anti and 13-cis,C=N syn isomers of retinal (6, 7). The 13-cis component of this dark-adapted form has a λ_{max} at 548 nm (8, 9). Only the all-trans species, which undergoes an isomerization to a 13-cis species during the primary photoreaction, appears to be capable of translocating protons across the membrane (5).

The photochemical reactions of the light-adapted form of bR have been extensively studied by using a variety of biophysical and biochemical methods (5, 10–12). Recently, site-specific mutagenesis has helped reveal the functional roles of specific amino acid residues in the molecular mechanism of light-driven proton transport (13–15). These studies have indicated that the correct interaction between Asp-85, Asp-212, Arg-82, Tyr-185, and the Schiff base is essential for the regulation of the function and color of bR (14–18). Many of these substitutions, which generally result in red-shifted chromophores, also show increased pK_a values for the purple-to-blue transition and a strongly perturbed photocycle.

In contrast to these studies, relatively little is known about the effects of single amino acid substitutions on dark- and light-adaptation reactions. Here, we present a detailed study of the dark- and light-adaptation reactions of a set of substitution mutants involving the above-mentioned amino acids, Arg-82, Asp-85, Tyr-185, and Asp-212, all of which appear to be located in the retinal binding pocket (14, 19, 20). Visible absorption measurements and chromophore extractions indicate that none of these mutants exhibit a normal pattern of retinal isomerization during light adaptation. In addition, light adaptation of all of these mutants involves an additional stable species that has a λ_{max} near 600 nm.

METHODS

Sample Preparation. The construction, expression, and purification of bacterioopsin mutants carrying single substitutions have been reported (21, 22). Apoproteins were regenerated with all-*trans*-retinal and reconstituted in vesicles with polar lipids from *Halobacterium halobium*, using a lipid-to-protein ratio of 1:1 (wt/wt) (23). All experiments were performed at room temperature. Samples were suspended at a concentration of ≈10 μM bR in a standard buffer consisting of 150 mM KCl, 30 mM sodium phosphate, and 0.025% NaN_3, and the pH was adjusted with microliter amounts of 0.1 M NaOH or 0.1 M HCl.

Retinal Extraction. For the chromophore extraction experiments, the mutant apoproteins were regenerated with a limiting amount of all-*trans*-retinal (1:3 molar ratio) and reconstituted in vesicles with lipids from *H. halobium* as described above. To ensure that no unreacted retinal was present, the vesicles were washed five times with 5% (wt/vol) bovine albumin (24). The retinal chromophore was extracted after dark adaptation for 72 hr followed by light adaptation for 10 min, according to a published procedure (25). The isomer composition was calculated by using the appropriate extinction coefficients (26).

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Abbreviations: bR, bacteriorhodopsin; ebR, bacteriorhodopsin produced from the expression of a synthetic wild-type gene in *Escherichia coli*; λ_{max}, wavelength of maximum absorption. bR mutants are designated by the wild-type amino acid residue (standard one-letter code) and its position number followed by the substituted amino acid residue. Thus, Y185F signifies the mutant in which the tyrosine at position 185 has been replaced by phenylalanine.

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Absorption Spectroscopy. Visible absorption spectra were recorded by using a UV–visible Shimadzu 2100 spectrometer equipped with a 60-mm integrating sphere in order to reduce absorption losses due to light scattering of the sample. Illumination was from a 150-W tungsten light source filtered through various glass filters as indicated below. For all measurements, vesicle suspensions of bR produced from the expression of a synthetic wild-type gene in *Escherichia coli* (ebR) and mutants (0.3–0.5 absorbance units) were kept in the dark for at least 72 hr prior to recording a spectrum.

The following sequence of reactions was determined for ebR and the mutants at room temperature.

(i) Light and dark adaptation as a function of pH. Light adaptation was performed by illumination for 2 min using a 505-nm long-pass glass filter (Ditric Optics, Hudson, MA). A set of absorbance spectra was recorded afterwards to follow the thermal dark adaptation of the sample and to determine if the light adaptation was reversible in the dark. Difference spectra of the dark-adapted minus the light-adapted sample were obtained.

(ii) Red light photoreaction of the dark-adapted state and subsequent illumination with blue light. Dark-adapted samples were extensively illuminated by using a 650-nm long-pass filter. The absorption spectra were recorded at different times during illumination. The samples were then illuminated with a 490-nm short-pass filter for 2 min, and the resulting absorption was measured.

(iii) Red light photoreaction of the light-adapted state. The same procedure was used as in step *ii*, except that the sample was light adapted prior to illumination with red light.

RESULTS

Tyr-185 Mutant. It has been observed (4) that unlike ebR, the Tyr-185 → Phe (Y185F) mutant exhibits a pH-dependent absorbance spectrum between pH 5 and 8, reflecting contributions from mainly two different chromophores. Titration experiments indicated a reversible transition between a red-shifted chromophore (λ_{max} near 600 nm) at low pH and a purple chromophore with λ_{max} at 550 nm at higher pH, with an apparent pK_a near 7.0. Fig. 1*A* shows the absorbance spectra of the dark- and light-adapted forms of Y185F at pH 7.5. Light adaptation of this mutant causes a red shift in the λ_{max}. However, the difference spectrum for this reaction shows a significant 20-nm shift to longer wavelength compared to ebR (Fig. 1*B*). In contrast to ebR, the dark minus light difference spectrum exactly matched the difference spectrum obtained by subtracting the spectrum of the low pH form of Y185F, which is blue, from the high pH form, which is purple (Fig. 1*C*). Thus, light causes the conversion of a purple form with a λ_{max} at 550 nm to a blue form with a λ_{max} near 600 nm and not to a 570-nm form, as occurs normally with light adaptation. It was also found that the amount of blue species formed with light was pH dependent (Fig. 1*C*), reaching a maximum at pH 7.5. Unlike ebR, where light adaptation occurred in less than 2 min, the light-adaptation reaction of Y185F was completed after 10 min. In the dark at pH 6, Y185F returned to its original dark-adapted state with a time constant of 15 hr compared to 30 min for ebR.

To examine the behavior of the blue species formed upon light adaptation, we exposed the dark- and the light-adapted samples to red light (see steps *ii* and *iii* in *Methods*). As shown in Fig. 1*D*, both samples formed the same amount of a species with a λ_{max} near 480 nm, although the sample that had been previously light adapted had an increased content of the blue species. The 480-nm species is most likely equivalent to pink membrane, which contains retinal in the 9-cis configuration and is formed upon illumination of deionized purple membrane with deep-red light (27–29). As previously observed for pink membrane formed from blue membrane (30), illumination of the pink form with 490-nm light caused a complete recovery of the original spectrum. However, the amount of pink membrane produced was not dependent on prior light adaptation, indicating that the blue species formed upon light adaptation of Y185F is not able to form pink membrane. This result was also obtained for shorter periods of red light illumination, eliminating the possibility that the slow decay of the blue species reduced the amount of pink species formed for the light-adapted case. We therefore conclude that the blue species formed by light adaptation of Y185F does not undergo any blue-to-pink reactions, indicating that this species is not identical to the low pH blue form of Y185F. One possible explanation is that the blue form produced upon light adaptation contains a retinal chromophore in a different configuration than that of the low pH blue form. We also note that ebR at pH 7.0, when subjected to the same illumination procedure, did not form a pink species.

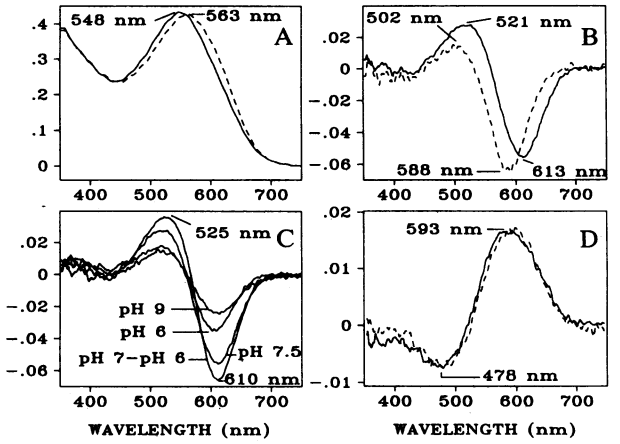

FIG. 1. (*A*) Absorption spectra of the dark-adapted (——) and light-adapted (– – –) states of Y185F at pH 7.5. In *B* and *C* the difference spectra of the dark-adapted minus the light-adapted states are shown for Y185F (——) and ebR (– – –) at pH 7.5 (*B*) as well as Y185F at pH 6.0, 7.5, and 9.0 (*C*). The difference spectrum of Y185F obtained by subtracting the dark-adapted absorption spectrum at pH 6.0 from that at pH 7.0 is also shown in *C*. The concentration was 0.45 absorbance unit at the λ_{max}. (*D*) Difference spectra of Y185F at pH 7.5 obtained as the difference of the dark-adapted spectrum and the one recorded upon illumination at 650 nm for 3 min (——) and obtained as the difference of the light-adapted spectrum and the one recorded upon illumination at 650 nm for 3 min (– – –). See text for details. The concentration measured in the dark for both experiments was 0.3 absorbance unit at the λ_{max}.

As shown in Table 1, the extraction of the retinal chromophore for the mutant Y185F reveals a pattern for the isomeric ratio, which was relatively constant between pH 5 and 8 and similar to wild type. Dark-adapted samples had a ratio of ≈2:1 for 13-cis to all-trans chromophore, whereas in the light-adapted samples the all-trans chromophore in-

Table 1. Retinal extraction of Y185F

pH	Ratio of 13-*cis*- to all-*trans*-retinal after dark adaptation	Ratio of 11-*cis*- to 13-*cis*- to all-*trans*-retinal after light adaptation (10 min)	Ratio of 13-*cis*- to all-*trans*-retinal after dark adaptation (48 hr)
5.2	62:38	0:14:86	
6.1	63:37	0:16:84	70:30
7.1	64:36	0:17:83	
8.1	61:39	1:26:73	67:33
9.2	63:37	14:30:56	
10.1	60:40	30:39:31	

The retinal extractions were performed on Y185F reconstituted into native membranes, using the procedure described in *Methods*.

creased to >80%. This indicates that the light-adapted blue species has a chromophore that is predominantly all-trans. Interestingly, there is no light-adaptation reaction detected at pH 5, yet there is a significant conversion of 13-cis to all-trans chromophore. We also note that above pH 8, the all-trans content for light-adapted samples decreased.

Arg-82 Mutants. The absorbance spectrum of R82Q at pH 6 has a λ_{max} at 584 nm, which does not shift upon light adaptation of the sample, most likely because the pK_a for the purple-to-blue transition is near pH 7 (18). Thus, at this pH this mutant is mainly in the blue form. The λ_{max} of this mutant shifts toward shorter wavelength values with increasing pH. At pH 8, the absorbance spectrum in the dark shows a λ_{max} at 548 nm that red shifts to 553 nm after light exposure (Fig. 2*A*). The dark minus light difference spectrum is similar to that obtained upon illumination of Y185F, which again reflects a purple-to-blue conversion of the mutant chromophore (Fig. 2*B*). No further λ_{max} shift is observed with additional illumination. In contrast to Y185F, the dark adaptation of R82Q is very fast, with a time constant of 6 min. Extended illumination of this mutant at pH 6 with 650-nm light shows the expected blue-to-pink transition (Fig. 2 *C* and *D*), and subsequent exposure to 490-nm light for 2 min totally restores the original spectrum (18). R82A has the same behavior as R82Q, except that the light–dark adaptation is even faster; it occurs in less than 3 min. Above pH 8.5, a transition to a form with a λ_{max} at 470 nm was observed upon illumination of R82A.

Asp-212 Mutants. It has been reported previously that the absorbance spectrum of D212E exhibits a reversed λ_{max} shift during light adaptation (21). In agreement, at pH 8 the light-adapted form is blue shifted (λ_{max} = 556 nm) compared to the dark-adapted form (λ_{max} = 566 nm) (Fig. 3*A*). The original dark-adapted spectrum is recovered after 3 hr in the dark. However, the dark minus light difference spectrum of D212E (Fig. 3*B*) is not simply the negative of the dark minus light difference spectrum of ebR (compare with Fig. 1*B*). Comparison of the spectra shows that the 610-nm band is upshifted by ≈20 nm relative to ebR. Once the sloping baseline is taken into account, the difference spectrum of this mutant is similar to that obtained for the blue-to-pink photoconversion created by irradiating a deionized blue membrane sample with deep-red light (dashed line, Fig. 3*B*), although the D212E photoproduct appears to be heterogeneous because of additional blue-shifted species. Thus, the main effect of light adaptation in D212E is the conversion of a blue species absorbing near 600 nm to a species absorbing around 480 nm.

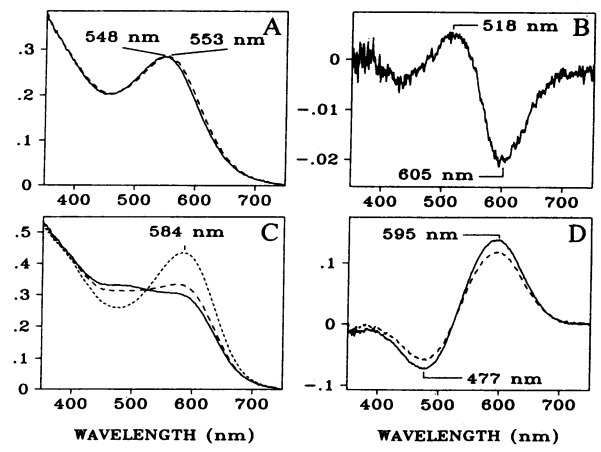

FIG. 2. (*A*) Absorption spectra of the dark-adapted (——) and light-adapted (– – –) states of R82Q at pH 8.0. (*B*) Difference spectrum of the dark-adapted minus the light-adapted state of R82Q at pH 8.0. The concentration was 0.3 absorbance unit at the λ_{max}. (*C*) Absorption spectra of R82Q at pH 6.0 after illumination with 650-nm light for 60 (– – –) and 90 (——) min, followed by illumination at 490 nm for 2 min (- - - -), which causes complete recovery of the original absorption spectrum. (*D*) Difference spectra of R82Q obtained from spectra recorded before and after illumination at 650 nm for 60 (– – –) and 90 min (——).

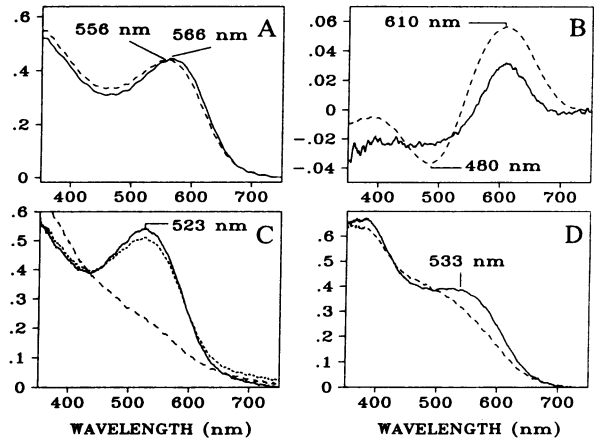

FIG. 3. (*A*) Absorption spectra of the dark-adapted (——) and light-adapted (– – –) states of D212E at pH 8.0. (*B*) Difference spectrum of the dark-adapted minus the light-adapted spectrum shown in *A* (——) and difference spectrum of a deionized purple membrane sample at pH 5.0 obtained from spectra recorded before and after illumination at 650 nm for 10 min (– – –). (*C*) Absorption spectra of the dark-adapted (——) and the light-adapted (– – –) states of D212A at pH 6.0 and spectrum of the light-adapted sample after dark adaptation for 15 hr (- - - -). (*D*) Absorption spectra of the dark-adapted (——) and the light-adapted (– – –) states of D212N at pH 6.0.

Retinal extractions of D212E under similar conditions used for the dark–light spectroscopic measurements reveal that the dark-adapted form consists mainly of all-*trans*-retinal with an ≈10% 13-cis component, which remains constant between pH 5 and 9 (Table 2). In contrast, light adaptation causes a conversion to a heterogeneous mixture consisting of all-*trans*-retinal along with smaller levels of 9-, 11-, and 13-*cis*-retinal. The amount of all-trans isomer decreases at higher pH values. We therefore conclude that D212E exhibits a pattern of chromophore isomerization during the dark–light adaptation that is very different from wild-type bR.

The D212A and D212N mutants also exhibited blue shifts during light adaptation. However, as shown in Fig. 3 *C*, this may reflect partial bleaching of the chromophore. In the dark, D212A slowly regenerates back the original pigment, whereas for D212N the purple color is not restored.

Asp-85 Mutants. The D85N and D85A mutants have red-shifted chromophores at pH 6 with λ_{max} at 590 and 610 nm, respectively. Light adaptation does not induce any changes in their absorbance spectra. However, upon extended illumination for 90 min with 650-nm light, D85N partially produced a pink form with λ_{max} at 480 nm (Fig. 4 *A* and *B*). Subsequent exposure of this sample to 490-nm light for 2 min

Table 2. Retinal extraction of D212E

pH	Ratio of 13-*cis*- to all-*trans*-retinal after dark adaptation	Ratio of 9-*cis*- to 11-*cis*- to 13-*cis*- to all-*trans*-retinal after light adaptation (10 min)
5.2	10:90	3:15:21:61
6.1	10:90	4:10:20:66
7.2	10:90	5:11:26:58
8.2	12:88	8:19:29:44
9.2	11:89	9:25:32:34

The retinal extractions were performed on D212E reconstituted into native membranes, using the procedure described in *Methods*.

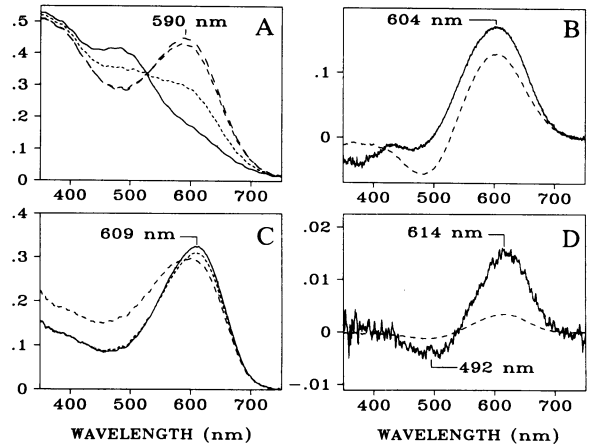

FIG. 4. (*A*) Absorption spectra of D85N at pH 6.0 after illumination with 650-nm light for 60 (- - - -) and 120 (——) min, followed by illumination at 490 nm for 2 min (– – –, second trace from top). The original absorption spectrum before illumination is also shown (– – –, top trace). (*B*) Difference spectra of D85N (– – –) and D85A (——) at pH 6 obtained from spectra recorded before and after illumination at 650 nm for 60 min. (*C*) Absorption spectra of the dark-adapted (——) and the light-adapted (- - - -) states of D85E at pH 6.0 and the dark-adapted state at pH 8.0 (– – –). (*D*) Difference spectra of the dark-adapted minus the light-adapted states of D85E at pH 6.0 (——) and pH 8.0 (– – –).

efficiently converted the pink form back to the original chromophore. The effect of long-wavelength illumination on D85A produced a pink form, as well as an increased absorbance at 370 nm reflecting the loss of chromophore (Fig. 4*B*). However, the sample also recovered its initial absorbance spectrum after illumination with light <490 nm. Titration of these two mutants results in a reversible pH-dependent transition (with a pK_a = 8.8 for D85N and 9.3 for D85A) from a blue to a 410-nm chromophore characteristic of a deprotonated Schiff base linkage (data not shown). These data and the lack of any purple species are in agreement with recent results (15, 31). The small differences from the previously reported pK_a values are most likely due to the use of a different reconstitution system.

Fig. 4*C* shows the absorbance spectra of D85E at pH 6 before and after light adaptation. At pH 6 the dark-adapted minus light-adapted difference spectrum of this mutant shows a maximum at 614 nm and a minimum at 492 nm (Fig. 4*D*). Thus, illumination induces a blue-to-pink transition qualitatively similar to the effect observed for D212E. At pH 8 the dark-adapted D85E mutant displays an absorbance spectrum with a maximum at 604 nm (Fig. 4*C*). This spectrum reflects contributions from three different chromophores absorbing near 610 nm (blue form), 560 nm (purple form), and 490 nm, respectively. After light adaptation at pH 8, a difference spectrum similar to pH 6 is obtained, except for a highly reduced amplitude (Fig. 4*D*). Thus, despite the presence of a purple form at this pH in D85E, normal light adaptation is not observed.

Extended illumination of D85E at 650 nm also forms a pink species that converts back to its original state upon illumination at 490 nm (data not shown). However, illumination of the sample above pH 8 with 650-nm light, which favors the equilibrium toward the 490-nm species, does not induce any further changes, suggesting that this species is different from the metastable pink membrane formed upon illumination with deep-red light at lower pH. Above pH 10, the 490-nm species converts to a chromophore that absorbs at 370 nm, reflecting the hydrolysis of the retinal–protein Schiff base linkage.

DISCUSSION

Earlier spectroscopic studies on bR mutants have focused mainly on light-adapted bR and its photocycle intermediates (1–4, 14–16, 18, 32–38). Many of these studies support a model of the retinal binding pocket originally proposed on the basis of Fourier transform IR spectroscopy and other spectroscopic measurements (14, 19). This model envisions the mutual interaction of the residues Tyr-185, Asp-212, Asp-85, and Arg-82 near the protonated Schiff base of retinal in light-adapted bR. Asp-85 was predicted to be the acceptor of the Schiff's base proton during M formation and Asp-212 was predicted to be the proximal proton donor to the Schiff base. A recent bR electron density map based on electron diffraction (20) supports many of these structural predictions, including the interaction of Asp-212 and Asp-85 with the Schiff base. Furthermore, Asp-212 appears to interact with Tyr-185 (20), possibly through a polarizable hydrogen bond (39).

Our present results demonstrate that mutants of all of these "active site" residues have drastically altered properties for light–dark adaptation. In contrast to normal light adaptation in bR, none of the mutants exhibited the characteristic 558 → 568-nm shift, which reflects the isomerization from a 13-cis,syn to an all-trans,anti configuration. Instead, the mutants exhibited reversible light adaptation involving a red-shifted (blue) species that behaves different from the blue species formed by acid titration or deionization of bR.

In the case of Y185F, R82A, and R82Q, light adaptation converts a purple form absorbing near 550 nm to a blue form absorbing near 600 nm. Previously, we have shown that at low pH a blue form of Y185F is produced in the dark, which behaves very similar to acid blue (4). However, the light-induced blue form of Y185F behaves differently; for example, it does not exhibit a red light-induced pink form. Retinal extraction demonstrates that the Y185F photoreaction involves mainly a 13-cis → all-trans isomerization at both low and high pH. One interesting possibility is that the species photoconverted has a 13-cis,anti chromophore, similar to the conformation of the N intermediate in the bR photocycle, whereas the blue form created upon illumination has an all-trans,syn chromophore. Both of these forms appear only transiently in bR during the photocycle of light-adapted bR_{568} (40) and dark-adapted bR_{548} (41), respectively. However, it is possible that these mutants act to stabilize isomeric forms of retinal that are normally not allowed in the retinal pocket.

In agreement with the above hypothesis, resonance Raman spectroscopy indicates that Y185F has an increased content of an N-like form (P. Rath, M.D., T.M., H.G.K., and K.J.R., unpublished results), which may be due to a defect in isomerization of the chromophore from 13-cis to all-trans during the photocycle (39). In native bR, N may exist at low concentration in equilibrium with bR_{568} (42). In analogy with light adaptation in native bR, which produces a double isomerization around the $C_{13} = C_{14}$ double bond and the C=N bond, such an isomerization of a 13-cis,anti chromophore would yield an all-trans,syn chromophore. A red-shifted all-trans,syn chromophore has been previously observed in the primary photoproduct of the 13-cis,syn form of dark-adapted bR_{548} (41). It has also been recently suggested that the N intermediate has a red-shifted photoproduct (43). Further studies using resonance Raman and Fourier transform IR spectroscopy should be able to test this hypothesis.

In the case of D85E and D212E, light adaptation causes a conversion from a blue species absorbing near 610 nm to a heterogeneous mixture of species absorbing at and below 480 nm. However, this photoreactive blue form is not likely to be the same as deionized or acid blue. In particular, irradiation with light near 500 nm is expected to have a greater quantum efficiency for conversion of pink back to blue and hence not produce an accumulation of a pink form. One possibility is that this altered blue form may also contain a stable all-trans,syn chromophore that isomerizes partially upon illumination to an all-trans,anti chromophore. The chro-

mophore extraction results indicate that in addition to all-*trans*-retinal, there is a mixture of 9-, 11-, and 13-*cis*-retinal chromophore, which could account for the broad blue-shifted band observed upon light adaptation. The all-trans component may have a protein conformation similar to that obtained by subjecting bR to a variety of external perturbations, including heating and dimethyl sulfoxide exposure (44).

In the case of the mutants D212A, D212N, D85A, and D85N, all substitutions that introduce a neutral residue near the Schiff base, there is a tendency for either light or elevated pH to cause a large blue shift, which most likely reflects a deprotonation of the Schiff base. These results can be understood if these mutants allow an increased accessibility of water into the active site. This might occur, for example, if water rather than Asp-212 acted as the proximal proton donor for the Schiff base, thereby catalyzing hydrolysis of the Schiff base bond.

CONCLUSIONS

The present study reveals that additional forms of bR are possible when key residues, which are part of the bR active site, are substituted. Light adaptation in Y185F produces a blue form that is distinct from the acid- and deionized-blue membrane. Although the chromophore configuration of this blue species at present is unknown, indirect evidence supports an all-trans,syn chromophore, which is normally not stable in native bR. This form may be produced from a double isomerization of a stable 13-cis,anti form absorbing near 550 nm. Further measurements using resonance Raman spectroscopy and Fourier transform IR difference spectroscopy will be necessary to test this hypothesis.

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