The Retinylidene Schiff Base Counterion in Bacteriorhodopsin*

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Previous studies of bacteriorhodopsin have indicated interactions between Asp-85, Asp-212, Arg-82, and the retinylidene Schiff base. The counterion environment of the Schiff base has now been further investigated by using single and double mutants of the above amino acids. Chromophore regeneration from bacteriorhodopsin proceeds to a normal extent in the presence of a single aspartate or glutamate residue at position 85 or 212, whereas replacement of both charged amino acids in the mutant Asp-85 → Asn/Asp-212 → Asn abolishes the binding of retinal. This indicates that a carboxylate group at either residue 85 or 212 is required as counterion for formation and for stabilization of the protonated Schiff base. Measurements of the pKₐ of the Schiff base reveal reductions of >3.5 units for neutral single mutants of Asp-85 but only decreases of <1.2 units for corresponding substitutions of Asp-212, relative to the wild type. Substitutions of Asp-85 show large red shifts in the absorption spectrum that are partially reversible upon addition of anions, whereas mutants of Asp-212 display minor red shifts or blue shifts. We conclude, therefore, that Asp-85 is the retinylidene Schiff base counterion in wild-type bacteriorhodopsin. In the mutant Asp-85 → Asn/Asp-212 → Asn formation of a protonated Schiff base chromophore is restored in the presence of salts. The spectral properties of the double mutant are similar to those of the acid-purple form of bacteriorhodopsin. Upon addition of salts the folded structure of wild-type and mutant proteins can be stabilized at low pH in lipid/detergent micelles. The data indicate that exogenous anions serve as surrogate counterions to the protonated Schiff base, when the intrinsic counterions have been neutralized by mutation or by protonation.

Bacteriorhodopsin (bR) is a transmembrane protein that functions as a light-driven proton pump in the purple membrane of Halobacterium halobium (1). The protein contains seven α-helical transmembrane segments and a molecule of all-trans-retinal, which is covalently linked to Lys-216 as a protonated Schiff base (PSB) (Fig. 1). The transport of protons involves a photochemical cycle that consists of at least five transient intermediates (4, 5). Recently, site-specific mutagenesis has identified several amino acid residues that are involved in proton translocation. The interactions between Asp-85, Asp-212, Arg-82, and the protonated Schiff base (cf. Fig. 1) were shown to be critically important for the regulation of function and absorption maximum of bR (6–11). In the recently proposed structural model of bR the side chains of Asp-85 and Asp-212 are approximately equidistant (~4 Å) from the PSB (2), and both residues appear to be deprotonated in the ground state (7, 12). Moreover, the N → H bond of the chromophore is oriented toward the extracellular side of the membrane (13, 14), allowing an interaction of the PSB with Asp-85 or Asp-212. Thus, either or both aspartate residues are candidates for being counterions to the PSB. In a previous study we showed that replacement of Asp-85 by neutral amino acids leads to a significant reduction in the pKₐ of the Schiff base to between 7 and 8.2 and suggested that Asp-85 serves as the primary counterion to the PSB (10). Arg-82 is presumably ionized as well in bR and is likely to interact with Asp-85 and/or Asp-212. In addition, there is evidence that Asp-85, Asp-212, and Arg-82 are involved in the proton release from the Schiff base in the early phase of the photocycle (10). Specifically, Asp-85 functions as the proton acceptor during M formation (7, 10, 15–17). Based on action spectra it was proposed that Asp-85 also becomes protonated during the purple to blue transition at low pH (9).

In the present work, we have used single and double substitution mutants of Asp-85, Asp-212, and Arg-82 to investigate the counterion environment of the PSB in greater detail (Fig. 1). Our results show that a single carboxylate group (Asp or Glu) at position 85 or 212 fulfills the requirement for folding and subsequent formation of a PSB in bR. Measurements of the pKₐ values of the SB show large decreases for neutral substitutions of Asp-85 (2–4 orders of magnitude), whereas corresponding mutants of Asp-212 reveal SB pKₐ values similar to that of the wild type. Because of the greater effects on the absorption spectrum and on the SB pKₐ, for neutral replacements of Asp-85 relative to Asp-212, we conclude that Asp-85 and the PSB show a stronger electrostatic interaction and that Asp-85 is the retinylidene Schiff base counterion in the ground state of bR. In the absence of an endogenous counterion, as is the case in the double mutant D85N/D212N, exogenous anions can serve as surrogate coun-

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‡ The abbreviations used are: bR, bacteriorhodopsin; eBr, bacteriorhodopsin prepared by expression of a synthetic wild-type gene in E. coli; PSB, the protonated Schiff base; SB, the unprotonated Schiff base; DA, the dark-adapted form of the chromophore; LA, the light-adapted form of the chromophore; DMPC, 1,2-dimyristoylphosphatidylcholine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate.

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ApaI-BglII restriction fragments containing the desired mutations
phy (21). The average yield of mutant apoproteins was 40-50 mg/lO
from crude E. coli membranes with an organic solvent mixture and
residue 212). For the double mutants R82Q/D85N and R82D/D85R,
sponding single mutations (18, 19). In each case a small
D212E, or D212N has been reported (6, 8, 10). Genes encoding the
regions carrying the mutations were verified by direct plasmid se-
were synthesized and ligated with the small
important role in regulating the wavelength of absorbance in
bacteriorhodopsin. The
nature of the anions (halides, perchlorate, and different carboxylic acids) greatly influences the absorption maxima of the mu-
and allow chromophore regeneration. The spectral prop-
properties of the D85N/D212N mutant are similar to those of the so-called acid-purple form of wild-type bR. The nature of the anions (halides, perchlorate, and different carboxylic acids) greatly influences the absorption maxima of the mutants. Large spectral shifts are observed for D85A (λ_{max} between 544 and 603 nm), D85E (λ_{max} between 560 and 607 nm), and D212N (λ_{max} between 494 and 562 nm) in the presence of different anions. This indicates that electrostatic interactions between the PSB and its counterions play an important role in regulating the wavelength of absorbance in bacteriorhodopsin.

EXPERIMENTAL PROCEDURES

Preparation of bR Mutants—The preparation of mutants containing the single substitutions R82Q, D85A, D85E, D85N, D212A, D212E, or D212N has been reported (6, 8, 10). Genes encoding the double mutants R82Q/D212N, D85E/D212E, D85E/D212N, D85N/D212N, and D85N/D212N were assembled using restriction fragments of previously constructed pSB02 vectors that carry the corresponding single mutations (18, 19). In each case a small Poul-BglII fragment (encoding the mutation at residue 82 or 85) was ligated to a large BglII-Poul fragment of pSB02 (encoding the mutation at residue 212). For the double mutants R82Q/D85N and R82D/D85R, Apal-BglII restriction fragments containing the desired mutations were synthesized and ligated with the small Poul-Apal fragment and the large BglII-Poul fragment of wild-type pSB02. The sequences of regions carrying the mutations were verified by direct plasmid sequencing via the dyeoxy method (20). The mutant genes were then introduced into the vector pPL1 as HindIII-EcoRI fragments (18) and expressed heterologously in Escherichia coli under the control of the lac operon (8, 19). All of the mutant apoproteins were extracted from crude E. coli membranes with an organic solvent mixture and purified to apparent homogeneity by DEAE-Trisacryl chromatography (21). The average yield of mutant apoproteins was 40-50 mg/10 g of freeze-dried membranes.

Regeneration and Characterization of bR Mutants—bR-like chromophores were regenerated from the apoproteins (16 μM) in a solution of 1% DMPC, 1% CHAPS, 0.2% SDS, and 1 mM sodium phosphate, pH 6.0, by the addition of all-trans-retinal (19). The kinetics of chromophore formation were measured at 20 °C in the presence of a >3-fold molar excess of retinal. Under these conditions the regeneration rate is independent of the retinal concentration (22). The absorbance increase at the λ_{max} of the dark-adapted chromophore was monitored. The effect of pH on the chromophore was studied in DMPC/CHAPS/SDS mixed micelles, and the pH was adjusted by adding microliter aliquots of 0.1-0.5 M NaOH or 0.1-2 M HSO₄. Extinction coefficients were determined by acid denaturation of each mutant in the dark to a give a chromophore with λ_{max} at 442 nm (23). The rate of absorption at the λ_{max} to the absorption at 442 nm after acidification to pH 1.9 was compared with that of wild-type bR. The extinction coefficient of bR was assumed to be 52,000 M⁻¹cm⁻¹ (22). The λ_{max} values of the chromophores were measured at 4 °C after dark adaptation followed by light adaptation for 3 min (250-watt projector lamp equipped with a 470-nm long-pass filter). Retinal was extracted from the chromophores after dark adaptation followed by light adaptation for 3 min, as described previously (23, 24).

Effects of Exogenous Salts on the Absorption Spectrum of bR Mutants—Solutions of sodium salts of halides, perchlorate, and different carboxylic acids (formate, acetate, tartrate, citrate, chloroacetate, dichloroacetate, and trichloroacetate) were prepared at 2× (0.1, 1, or 4 M) concentrations. The salt solutions were titrated with their respective acids to give a pH of 6.0 upon dilution to 1×. The mutants were regenerated as described above and the individual 2× salt solutions were then added. In the case of the mutant D85N/D212N the chromophore was directly regenerated in the presence of different salts. All measurements were taken at a protein concentration of 4 μM in 1% DMPC, 1% CHAPS, 0.2% SDS, and 1 mM sodium phosphate at pH 6.0 ± 0.05.

Determination of the pK_{a} of the Schiff Base—The mutant proteins (6.4 μM) were prepared as described above in the absence or presence of 2 M NaCl. Titrations were carried out in steps of 0.1-0.3 pH units, and following complete equilibration (~3 min) pH readings and absorption spectra were recorded for each point. The pH-induced absorbance changes were determined from difference spectra as follows. A reference spectrum was recorded at a pH where the pigment was completely converted to the protonated form. This spectrum was subtracted from each of the spectra recorded at higher pH values, and the amount of titrated pigment was determined from the absorbance increase of the deprotonated SB at 365 nm (for bR, R82Q, D85E, D212A, D212E, D212N, and R82Q/D212N or 405 nm (for D85A, D85N, and D85N/D212N). In the case of R82D/D85R and R82Q/D85N the transition to the 405-nm form was incompletely separated from that to the 365-nm form. Thus, the amount of titrated pigment was determined from the absorbance increase at 382 nm, the isosbestic point of the two species. To obtain the apparent pK_{a}, the absorbance change ΔA was plotted versus pH. The following three-parameter curve (25) was then fitted to the points.

\[
\Delta A = \Delta A_{1/2} \left[ 1 + 10^{pK_a - \text{pH}} \right]
\]

The three parameters were: ΔA_{1/2}, the total absorbance change of the deprotonated SB; n, the number of protons involved in the transition; and pK_{a}, the midpoint of the titration.

RESULTS

I. Effects of Substitutions of Asp-85 and Asp-212 on Chromophore Regeneration

Substitutions of Asp-85 or Asp-212 Alter the Kinetics of Chromophore Formation—We have reported previously (6) that substitution of Asp-85 or Asp-212 by Asn slows down the rate of chromophore regeneration (t_{1/2} = 41 and 31 min, respectively; Table I), compared with bR (t_{1/2} = 1 min). A similar effect was noticed for the mutant D212E (t_{1/2} = 38 min), whereas for D85E (t_{1/2} = 0.2 min) the rate was increased over the wild type. Striking differences in the kinetics are also observed with double mutants of Asp-85 and Asp-212 (Fig. 2; Table I). The regeneration process for D85N/D212E (t_{1/2} = 390 min) is slowed by more than 2 orders of magnitude compared to the wild type. On the other hand D85E/D212N (t_{1/2} = 0.5 min) and D85E/D212E (t_{1/2} = 2.8 min) reveal relatively normal regeneration times, suggesting that Glu-85...
TABLE I

Regeneration properties, absorption maxima, and isomer compositions of the chromophores of Asp-85, Asp-212, and Arg-82 mutants

| Mutation | Chromophore   | Regeneration | Extinction | Absorption maximum | Retinal isomer composition |
|----------|---------------|--------------|------------|--------------------|---------------------------|
|          | regeneration  | extent        | coefficient| DA                 | LA                        |
|          | (t0) min     | %            | M⁻² cm⁻¹  | 13-cis             | 9-cis                     |
|          |               |              | nm        | %                  | %                        |
| ebR      | 1.0           | 84           | 52,000    | 551                | 60 40                     |
| R82Q     | 3.2           | 63           | 56,200    | 575                | 50 50                     |
| D85E     | 0.2           | 75           | 56,400    | 597                | 51 49                     |
| D85N     | 41            | 84           | 54,700    | 595                | 36 64                     |
| D212E    | 38            | 74           | 58,400    | 584                | 9 91                      |
| D212N    | 31            | 75           | 52,700    | 582                | 22 78                     |
| R82Q/D85N| 780           | 40           | 50,700    | 597                | 16 84                     |
| D85N/D212N| 73            | 90           | 52,500    | 582                | 22 78                     |
| R82Q/D212N| 11            | 75           | 50,400    | 584                | 9 91                      |
| D85E/D212E| 2.8           | 76           | 60,400    | 584                | 9 91                      |
| D85E/D212N| 0.5           | 67           | 53,000    | 584                | 9 91                      |
| D85N/D212E| 190           | 81           | 53,100    | 584                | 9 91                      |
| D85N/D212N| 220           | 61           | ND        | 559                | 15 85                     |

* Measured at 20 °C with all-trans-retinal.
* The value given is at pH 5.0, since the chromophore exists in an equilibrium of protonated and unprotonated forms near neutral pH (see text).
* The properties were measured under standard conditions in the presence of 2 M NaCl.
* Calculated with ε = 52,000 M⁻¹ cm⁻¹.
* The value could not be determined, as no denaturation is observed at pH 1.9 (see text).

FIG. 2. Time course of chromophore regeneration for double mutants of Asp-85, Asp-212, and Arg-82. The mutant apoproteins were regenerated at 20 °C by the addition of excess all-trans-retinal, as described under "Experimental Procedures." The absorbance increase at the λmax of the dark-adapted chromophore was recorded. This was replotted after determination of the final absorbance. The corresponding tₐ values are listed in Table I.

Chromophore Regeneration Requires a Carboxylate Group at Position 85 or 212—Studies with the mutants D212N and D85N have shown that a single aspartate residue at either position 85 or 212 is sufficient to form a bR-like chromophore (6). A normal extent of chromophore regeneration between 556 and 597 nm (Table I) is also observed for the double mutants D85E/D212N and D85N/D212E, which contain a single glutamate residue at position 85 or 212, respectively. However, removal of both aspartate residues in the double mutant D85N/D212N prevented chromophore formation (<2%). This demonstrates that formation of a protonated retinylidene Schiff base in bR requires a carboxylate group (Asp or Glu) at either position 85 or 212.

II. Effects of Double Mutants of Asp-85, Asp-212, and Arg-82 on the Absorption Spectrum

Properties of the Dark- and Light-adapted Chromophores—It has been reported previously that single mutants of residues Asp-85, Asp-212, and Arg-82 generally show red-shifted chromophores at pH 6 relative to ebR (6, 8, 9) and exhibit altered dark to light adaptation reactions (Table I; Ref. 26). Consistent with this observation, all double mutants display in the dark-adapted state (continuous lines) followed by light adaptation for 3 min (broken lines). The corresponding λmax values are listed in Table I.
increased extinction, due to conversion of the 13-cis/all-trans chromophore into essentially 100% all-trans-retinal (cf. Fig. 24 of Ref. 23). Instead, light adaptation results in a reduced shift to longer wavelength (Fig. 3, A and B) or in a shift to shorter wavelength (e.g. in D85E/D212N and D85N/D212E; Table I). The light minus dark difference spectra of these mutants reveal a decrease in the absorbance of the chromophore and formation of blue-shifted species with \( \lambda_{\text{max}} \) between 420 and 460 nm, except for R82Q/D212N. Retinal extraction experiments indicate a relatively normal 13-cis to all-trans ratio in both the dark- and light-adapted states of R82Q/D212N (Table I). However, the other double mutants contain predominantly all-trans-retinal in the DA form (69–94%). Upon illumination the retinal isomer ratio stays constant in R82Q/D85N, whereas in D85E/D212E, D85E/D212N, and D85N/D212E significant fractions of cis isomers are formed. The lower extinction coefficients of the cis-retinal isomers can explain in part the light-induced decreases in absorbance and spectral blue shifts seen for these double mutants.

**Neutral Substitutions of Asp-85 Show Larger Spectral Red Shifts Compared with Asp-212 Substitutions**—Spectrometric titrations in DMPC/CHAPS/SDS micelles have shown that the mutant D212N displays a \( \lambda_{\text{max}} \) near 558 nm between pH 5 and 10, whereas D85N has a red-shifted chromophore with \( \lambda_{\text{max}} \) near 590 nm at this pH range (9, 10). In Fig. 4 the visible absorption maximum is plotted as a function of pH for the double mutants R82Q/D85N and R82Q/D212N. In comparing the two mutants, it is evident that between pH 4 and 8 the chromophore of R82Q/D85N is red-shifted by approximately 20 nm relative to R82Q/D212N. Both double mutants display shifts toward shorter wavelengths in the acidic and alkaline pH range. The increased spectral red shifts observed upon replacement of Asp-85 indicate that this residue exerts a greater electrostatic effect on the protonated Schiff base imine in bR, compared with Asp-212.

**III. Exogenous Anions as Counterions to the Protonated Schiff Base**

**Properties of the Double Mutant D85N/D212N in the Presence of Salts**—In the presence of different salts, chromophore regeneration is restored in the double mutant D85N/D212N. Fig. 5 shows that a maximum of 68% regeneration of the chromophore occurs in the presence of 3 M NaCl. The absorption maximum at 558 nm remains constant in the concentration range tested. Absorption spectra of the dark- and light-adapted forms of D85N/D212N in 2 M NaCl are displayed in Fig. 3D. Light adaptation of this mutant results in a minor red shift (\( \lambda_{\text{max}} \) 562 nm) and a small increase in the extinction.

**Fig. 4. pH dependence of the absorption maximum of R82Q/D85N (○) and R82Q/D212N (●).** The mutants were prepared in the absence of NaCl and titrated, as described under "Experimental Procedures." The \( \lambda_{\text{max}} \) of the dark-adapted chromophore is plotted as a function of pH. The measured absorbance of the chromophore at pH 6 was 0.73 and 0.65, respectively.

**Fig. 5. Extent of chromophore regeneration of D85N/D212N in the presence of varying NaCl concentrations.** The mutant was regenerated at pH 6 in the presence of NaCl, as described under "Experimental Procedures." The chromophore existed as a mixture of protonated and unprotonated forms. The proportion of the unprotonated form was <20% at all concentrations. The regeneration extent was calculated based on the absorbance at pH 5, where the chromophore was completely converted to the protonated form.

**Fig. 6. pH dependence of the absorption spectrum of D85N/D212N in the presence of 2 M NaCl.** Spectra were recorded at several pH values as indicated. At alkaline pH the PSB shows a reversible transition to a deprotonated SB with \( \lambda_{\text{max}} \) at 402 nm. Further alkalinization leads to the formation of a deprotonated SB with \( \lambda_{\text{max}} \) at 365 nm (see text). This conversion proceeds through an isosbestic point at 382 nm. At acidic pH the chromophore remains purple. The broken line shows the absorption spectrum at pH 1.5.

Retinal extraction experiments reveal for the DA chromophore an all-trans to 13-cis ratio of 85:15, which remains unchanged upon illumination (Table I).

The effect of pH on the absorption spectrum of D85N/D212N in 2 M NaCl is shown in Fig. 6. At low pH the chromophore of this mutant remains purple even at pH 1.5 (\( \lambda_{\text{max}} \) 554 nm; broken line in Fig. 6) and does not convert to a 442-nm absorbing species (free PSB). At alkaline pH the PSB chromophore reversibly titrates to a species with \( \lambda_{\text{max}} \) at 402 nm, representing a deprotonated SB (see below). A single isosbestic point at 460 nm is observed for this transition. Besides alkalinization, decreasing the NaCl concentration from 2 M to 0.5 M also shifts the equilibrium toward the deprotonated form (Fig. 7). The experiment indicates that in the presence of NaCl protonation of the SB is facilitated in this mutant. This effect is likely due to a specific interaction of chloride anions with the PSB.

**The Presence of Anions Affects the Absorption Spectrum of Asp-85, Asp-212, and Arg-82 Mutants**—According to the hypothesis that chloride anions interact with the PSB in D85N/D212N, the absorption spectrum of this and other mutants that alter the counterion environment should be sensitive to the type of anion present. This prediction was tested by measuring the \( \lambda_{\text{max}} \) values of mutants of Asp-85, Asp-212, and Arg-82 in the presence of sodium salts of halides, perchlorate, and different carboxylic acids (cf. "Experimental Procedures"). Control experiments show that the various anions...
have no effect on the absorption maximum of wild-type ebR ($\lambda_{\text{max}}$ at 551 or 552 nm; Table II and Fig. 8A). The mutants D85A, D85E, and D85N all display large shifts in the $\lambda_{\text{max}}$ (between 30 and 60 nm) in the presence of anions. The extreme $\lambda_{\text{max}}$ values for D85A are 544 nm in dichloroacetate and 603 nm in citrate, for D85E 560 nm in tartrate and 607 nm in trichloroacetate, and for D85N 568 nm in dichloroacetate and 597 nm in tartrate. The magnitude of the spectral shifts in D85A and D85N depends on the anion concentration (Table II). For D85A and D85E blue shifts in the $\lambda_{\text{max}}$ from ~600 nm to near the wild-type value are observed in several of these salts, indicating that solution anions substitute for the protein counterion.

Marked variations in the absorption maximum (between 30 and 70 nm) are also observed for neutral substitutions of Asp-212 upon addition of different anion solutions. However, the $\lambda_{\text{max}}$ values seen in the presence of salts are generally blue shifted relative to the wild type. For D212A the $\lambda_{\text{max}}$ values range from 511 nm in trichloroacetate to 541 nm in chloride and for D212N from 494 nm in dichloroacetate to 562 nm in tartrate and citrate (Table II). Both mutants already show shifts in the $\lambda_{\text{max}}$ at low salt concentrations. In the case of the D212E and R82Q mutants the anion-induced spectral shifts are smaller. The $\lambda_{\text{max}}$ for D212E varies narrowly between 566 nm in dichloroacetate and 582 nm in citrate and for R82Q between 560 nm in fluoride and 582 nm in trichloroacetate. Upon regeneration in the presence of different solute anions, the double mutant D85N/D212N displays extreme $\lambda_{\text{max}}$ values at 544 nm in iodide and at 564 nm in bromide (Table II and Fig. 8F).

The absorption maxima of the mutants R82Q, D85A, D85E, D212A, D212N, and D85N/D212N vary significantly in the presence of halides (Fig. 8 and Table II). For R82Q and D85E a direct correlation between a red shift in the $\lambda_{\text{max}}$ and an increase in the ionic radius is observed in the order $F^- < Cl^- < Br^- < I^-$. However, the mutants D85A, D212A, D212N, and D85N/D212N do not show such a relationship. For example, in each mutant the $\lambda_{\text{max}}$ value in the presence of iodide is less than that in the presence of chloride.

The effect of different monovalent cations on the absorption spectrum of the D85A mutant was tested as well. Fig. 9 shows that in the presence of 1 M LiCl, NaCl, KCl, or RbCl, this mutant displays identical $\lambda_{\text{max}}$ values at 575 or 576 nm. This observation supports the idea that the salt-induced spectral shifts in these mutants are caused by anions.

Anions Stabilize the PSB Chromophore at Low pH—The absorption spectra of wild-type ebR and mutants in DMPC/CHAPS/SDS mixed micelles display at acidic pH a transition to a species with $\lambda_{\text{max}}$ at 442 nm (broken lines in Fig. 10), normally occurring between pH 4 and 2 (23, 27). This conversion represents the formation of a free PSB due to denaturation of the protein. Fig. 10, A and B, shows that this transition is not observed in the presence of different salts at 2 M concentrations. Instead, the chromophores of ebR and the D85N mutant display a purple or blue color at pH 1.3. For ebR the $\lambda_{\text{max}}$ values are 593, 576, 573, and 566 nm in NaNClO$_4$, NaI, NaBr, and NaCl, respectively. These anion-induced blue shifts in the sequence ClO$_4^- < I^- < Br^- < Cl^-$ are identical to those observed upon formation of the acid-purple state of purple membrane (28). For D85N the blue shifts seen in the presence of these salts are generally larger, and the sequence differs (Fig. 10B). The experiment indicates that at low pH solution anions can stabilize the PSB of the folded protein by electrostatic interaction.

**Table II**

| Anion          | Absorption maximum of the pigment |
|----------------|----------------------------------|
|                | ebR | R82Q | D85A | D85E | D85N | D212A | D212E | D212N | D85N/D212N |
| Water          | 561 | 575  | 602  | 597  | 595  | 540   | 584   | 560   | ND         |
| Fluoride       | 561 | 560  | 602  | 561  | 592  | 540   | 581   | 550   | 558        |
| Chloride       | 561 | 563  | 592 (554) | 563 | 594  | 541 (540) | 579  | 548  | 560        |
| D85A           | 561 | 568  | 563 (555) | 568 | 595  | 538 (557) | 581  | 544  | 564        |
| D85E           | 561 | 573  | 565 (559) | 594 | 588  | 519 (522) | 577  | 535  | 544        |
| Perchlorate    | 551 | 575  | 564 (558) | 606 | 584  | 514 (519) | 579  | 529  | 546        |
| Chlorate       | 551 | 565  | 547  | 569  | 586  | 526 (527) | 574  | 505  | 548        |
| Dichloroacetate| 551 | 568  | 544  | 599  | 586  | 513 (517) | 566  | 494  | 550        |
| Trichloroacetate| 551 | 582  | 600  | 607  | 579  | 514 (514) | 580  | 507  | 57*        |
| Formate        | 551 | 568  | 596  | 564  | 593  | 535 (534) | 580  | 548  | 551        |
| Acetate        | 552 | 571  | 601  | 563  | 566  | 526 (522) | 581  | 552  | 555        |
| Tartrate       | 552 | 564  | 602  | 560  | 597  | 522 (531) | 581  | 562  | 553        |
| Citrate        | 552 | 564  | 603  | 561  | 596  | 535 (534) | 582  | 562  | 550        |

*The values listed in parentheses were measured in 2 M salt.

*The values listed in parentheses were measured in 50 mM salt.

*No chromophore regeneration is observed (see text).

*The chromophore is unstable ($t_\text{a}$, of bleaching ~20 h).
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FIG. 8. Effect of halides on the absorption spectrum of Asp-85, Asp-212, and Arg-82 mutants. A, wild-type ebR; B, R82Q; C, D85A; D, D85E; E, D212N; F, D85N/D212N. The pigments were prepared in 0.5 M sodium salts of halides, as described under "Experimental Procedures." The spectra are labeled from top to bottom in order of decreasing chromophore absorbance. The corresponding \( \lambda_{max} \) values are listed in Table II. The absorbance scale for A–D refers to the top curve only. The scale of the other spectra has been reduced by 45% to allow better presentation.

IV. Effects of Substitutions of Asp-85, Asp-212, and Arg-82 on the pK of the Schiff Base

The Apparent SB pK, of ebR is 11.3—To evaluate the interaction between the Schiff base and its environment, the SB pK, was determined for wild-type ebR and mutants. Spectrometric titration of dark-adapted ebR in DMPC/CHAPS/SDS mixed micelles results in a reversible shift of the \( \lambda_{max} \) from 551 nm at pH 6 to 544 nm at pH 10 (Fig. 11). Between pH 10 and 12 the purple chromophore converges to a species with \( \lambda_{max} \) at 365 nm. This transition proceeds through an isosbestic point at 410 nm and is complete at pH 11.9 (Fig. 11). If the alkaline system at pH 11.6 is rapidly neutralized to pH 6, several samples were acid denatured at pH 1.9 (23), following alkalinization to between pH 11 and 12. Fig. 11 (inset) shows that the 442-nm absorbance of the free PSB observed at pH 1.9 is identical, starting from samples at pH 6, 11, or 11.3. Above pH 11.3 a minor portion of the chromophore becomes hydrolyzed (~22% at pH 11.9). This indicates that the 365-nm absorbing species formed at alkaline pH represents essentially a deprotonated SB. The high pH, however, induces partial denaturation of the protein, thereby preventing complete recovery of the purple chromophore upon lowering the pH to around neutral. Fig. 12A shows the absorbance increase of the deprotonated SB of ebR as a function of pH. Analysis of the titration data (cf. "Experimental Procedures") yields an apparent SB pK, of 11.3 and a stoichiometry of 2.5 protons (Table II), indicating that the transition is cooperative. It is likely that deprotonation of the SB is induced by the titration of other protein groups, thereby causing denaturation.

The SB pK, Is Significantly Decreased in the Absence of a Carboxylate Group at Residue 85—Analogous spectrometric titrations were carried out for single and double mutants of
Fig. 11. Formation of a deprotonated Schiff base in wild-type eB R at alkaline pH. Absorption spectra were recorded in steps of 0.1–0.3 pH units (cf. Fig. 12A). Only spectra at the indicated pH values are shown. Between pH 10 and the PSB converts to a deprotonated SB with \( \lambda_{\text{max}} \) at 365 nm. This transition involves an isosbestic point at 410 nm. Neutralization of the alkaline system at pH 11.6 results in the partial recovery of the purple chromophore, whereas in D212A (Fig. 12B) the SB \( pK_a \) is slightly deprotonated SB in these mutants is cooperative, except in D212A, where it involves a single proton.

Table III

| Mutation     | \(-2 \text{ M NaCl}\) | \(+2 \text{ M NaCl}\) |
|--------------|------------------------|------------------------|
| \( pK_a \)   | \( n^o \)              | \( pK_a \)              | \( n^o \)              |
| eB R         | 11.26 ± 0.01           | 24.96 ± 0.01           | 2.77 ± 0.01            |
| R82Q         | 11.17 ± 0.01           | 21.22 ± 0.01           | 1.80 ± 0.01            |
| D85A         | 7.57 ± 0.01            | 1.05                  | 8.03 ± 0.01            |
| D85E         | 10.99 ± 0.03           | 1.48                  | 10.94 ± 0.02            |
| D85N         | 6.71 ± 0.01            | 0.91                  | 7.04 ± 0.01            |
| D212A        | 10.07 ± 0.01           | 1.06                  | 10.45 ± 0.03            |
| D212E        | 10.46 ± 0.03           | 1.51                  | 10.70 ± 0.03            |
| D85N/D212N   | 11.06 ± 0.01           | 1.34                  | 11.05 ± 0.02            |
| R82D/D85R    | 7.48 ± 0.01            | 1.00                  | 8.34 ± 0.03            |
| R82Q/D85N    | 9.43 ± 0.02            | 0.92                  | 9.68 ± 0.01            |
| R82Q/D212N   | 11.25 ± 0.01           | 2.26                  | 10.86 ± 0.03            |
| D85N/D212N   | 7.06 ± 0.01            | 2.77 ± 0.01            |

* Number of protons involved in the transition from protonated to deprotonated SB.

The \( pK_a \) values were determined in the absence or presence of NaCl, as described under “Experimental Procedures.”

The \( pK_a \) values of Asp-85, Asp-212, and Arg-82 mutants are listed in Table III. In R82Q, D85E, D212N (Fig. 12B), and R82Q/D212N formation of a deprotonated SB (\( \lambda_{\text{max}} \) at 365 nm) occurs with a \( pK_a \) comparable with the wild type, whereas in D212A (Fig. 12B) and D212E the SB \( pK_a \) is slightly decreased to 10.1 and 10.5, respectively. The transition to a deprotonated SB in these mutants is cooperative, except in D212A, where it involves a single proton.

Mutants that lack a carboxylate group at residue 85, namely D85A, D85N, D85N/D212N, R82Q/D85N, and R82D/D85R, display between pH 5 and 10 reversible transition from protonated to deprotonated SB chromophore (cf. Fig. 6 for D85N/D212N). In each case, the titration proceeds through an isosbestic point and involves a single proton (Table III), presumably the one at the Schiff base. The deprotonated SB in these five mutants has a \( \lambda_{\text{max}} \) near 405 nm and shows the characteristic features of the M intermediate of the photocycle (29). Above pH 10.5 a chromophore with \( \lambda_{\text{max}} \) at 365 nm is formed (Fig. 6), analogous to the transition observed in the case of eB R (Fig. 11) and all other mutants (e.g. R82Q, D212N). The transition between the two deprotonated SB species is cooperative (\( n > 1.5 \)) and proceeds through an isosbestic point at 382 nm (Fig. 6). The \( pK_a \) of SB deprotonation for mutants that lack a carboxylate group at residue 85 was determined based on the absorbance increase at either 405 or 382 nm (cf. “Experimental Procedures”). Analysis of the titration data for D85N (Fig. 12C) and D85A yields SB \( pK_a \) values of 6.7 and 7.6, respectively (Table III). Similar values have been reported previously for these mutants under slightly different experimental conditions (10). The SB \( pK_a \) for D85N/D212N was measured in 0.5 M NaCl, as the chromophore of this mutant becomes unstable at low salt concentrations. The titration data reveal a \( pK_a \) of 7.1, similar to the D85N single mutant. The SB \( pK_a \) is also decreased in the double mutants R82D/D85R and R82Q/D85N (Fig. 12D); the \( pK_a \) values determined are 7.5 and 9.4, respectively. The results show that in the absence of a carboxylate group at residue 85 the SB \( pK_a \) is lowered from 11.3 in eB R to between 6.7 and 9.4 in single and double mutants.

The SB \( pK_a \) of Asp-85, Asp-212, and Arg-82 Mutants Is Affected by Salt—The SB \( pK_a \) of the SB of wild-type bR decreases with increasing ionic strength, indicating that the Schiff base senses the negative charge at the membrane surface (30, 31). The decrease of the SB \( pK_a \) is in the order of one pH unit at molar salt concentrations and originates from unspecific screening of the negative surface charge by the salt. Superimposed on this effect there may be an increase of the SB \( pK_a \) in certain mutants due to specific binding of anions near the PSB, thereby stabilizing the protonated state. In the case of wild-type eB R in DMPC/CHAPS/SDS mixed micelles the apparent SB \( pK_a \) is essentially identical in the presence and absence of NaCl (Fig. 12A), presumably because deprotonation of the SB at high pH is associated with unfolding of the protein. In the mutants D85E and D212N the SB \( pK_a \) also remains unchanged in the presence of salt (Table III).
As suggested by the experiment shown in Fig. 7, the pKₐ of the SB in the D85N/D212N mutant is raised by 0.3 units upon increasing the NaCl concentration from 0.5 to 2 M. A comparable effect of NaCl on the SB pKₐ is also observed for D85A, D85N (Fig. 12C), D212A, D212E, and R82D/D85R. In each mutant the SB pKₐ of the SB is increased by 0.2–0.9 units in 2 M NaCl (Table III), indicating that the specific effect due to binding of chloride anions dominates the surface charge effect. The higher SB pKₐ in the presence of salt demonstrates that solution anions interact electrostatically with the PSB of these mutants.

Single and double mutants in which Arg-82 has been neutralized by Gln show a transition to a deprotonated SB that is decreased by 0.4–0.8 pH units in 2 M NaCl. Thus, the SB pKₐ values of R82Q, R82Q/D212N, and R82Q/D85N (Fig. 12D) are lowered to 10.6, 10.9, and 8.7, respectively, in the presence of salt (Table III). In these three mutants the surface charge effect seems to dominate. Removal of the positively charged Arg-82 could lead to increased surface charge effects compared with other mutants, if Arg-82 is accessible from or located close to the extracellular surface, as suggested (2). Alternatively, an increase of the SB pKₐ is not observed, since a specific chloride-binding site involving Arg-82 has been eliminated in these mutants.

**DISCUSSION**

In the present study we have used single and double mutants of residues Asp-85, Asp-212, and Arg-82 to examine the interactions between these groups and the retinylidene Schiff base in bR (Fig. 1). It has been reported previously (6, 10) that substitution of either Asp-85 or Asp-212 singly by Asn or Ala does not affect the ability of the resulting apoproteins to fold, bind retinal, and form a characteristic bR-like chromophore. The extent of chromophore regeneration is also decreased by 0.4–0.8 pH units in 2 M NaCl (Table III), indicating that the specific effect due to the titration of other residues, thereby causing partial denaturation of the protein. The apparent SB pKₐ of 11.3 for ebr in DMPC/CHAPS/SDS mixed micelles may be compared with the corresponding pKₐ of 13.3 for bR in purple membrane (32), which was shown to directly reflect titration of the Schiff base imine (33). Identical experiments with the D212N and D212A mutants reveal that the SB pKₐ is decreased by <1.2 units relative to the wild type upon substitution of the negatively charged Asp-212 (Fig. 12B). Single and double mutants that lack a carboxylate group at residue 85 show a reversible transition from protonated to deprotonated SB with pKₐ values between 6.7 and 9.4. In each case, the unprotonated SB displays a λₘₐₓ near 405 nm, thereby reflecting the protein-chromophore interactions at the M state of the photocycle, where Asp-85 is known to be non-ionized (protonated) (7, 16, 17). The titration data show that in D85N and D85A the SB pKₐ is strikingly reduced to 6.7 and 7.6, respectively, as reported previously (10). In the double mutants D85N/D212N, R82D/D85R, and R82Q/D85N the SB pKₐ of the SB is also lowered to between 7.1 and 9.4 (Table III). In contrast to the reversible transition of ebr at high pH, which probably involves unfolding of the protein, the reversible transition at lower pH to the 405-nm state in mutants with a neutral residue at position 85 is noncooperative. Similar noncooperative titrations have been observed in related systems with strongly reduced SB pKₐ values, such as bR regenerative with a retinal analogue (30), halorhodopsin (44), or octopus metarhodopsin (25). The close correspondence between the SB pKₐ values of the D212N mutant and ebr, as well as between those of the D85N/D212N and D85N mutants, indicates a minor role for Asp-212 in regulating the pKₐ of the SB. In contrast, the large decrease in the SB pKₐ observed upon substitution of Asp-85 implies that the high pKₐ of wild-type bR is largely due to stabilization of the PSB by the carboxylate group of Asp-85. The carboxylate group of the Schiff base counterion is the source of a negative electrostatic potential. Reducing this potential should cause a red shift in the absorption spectrum, besides decreasing the SB pKₐ of the SB (34). Consistent with this prediction, the λₘₐₓ values of the D85N and D85A mutants are red shifted to 595 and 602 nm, respectively. On the other hand, neutral substitutions of Asp-212 display λₘₐₓ values of 560, 540, and 505 nm for D212N, D212A, and D212Q, respectively. This observation supports the idea that the carboxylate group of Asp-85 exerts greater electrostatic influence on the PSB in the ground state of bR, compared with Asp-212.

It was of interest to study the effects of the double mutants R82Q/D85N and R82Q/D212N on the absorption maximum and the SB pKₐ, since Arg-82 has been proposed to form a salt bridge with Asp-85 and/or possibly Asp-212 (7–10, 12). The results show that Asn-85 is also red shifted relative to Asn-212 when combined with a Gln-82 substitution (Fig. 4). Assuming an interaction between Asp-85 and Arg-82, the λₘₐₓ as well as the SB pKₐ would be expected to have comparable values in both D85N and R82Q/D85N. The fact that R82Q/D85N shows a blue-shifted absorption maximum and significantly higher SB pKₐ relative to D85N could suggest an interaction between Arg-82 and Asp-212 in the D85N mutant, which may not occur in wild-type bR. Alternatively, it is possible that in D85N the positively charged guanidinium group of Arg-82 is located close to the PSB and causes lowering of its pKₐ. Since compensatory effects in the mutants cannot be excluded, it remains unclear whether Arg-82 interacts with Asp-85 and/or Asp-212 in the ground state of bR.

The spectral changes observed for the mutants in the pres-

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1 T. Marti and H. G. Khorana, unpublished observations.
ence of halides, perchlorate, and different carboxylic acids also point to a primary role of Asp-85 as counterion to the PSB. In D85A and D85E several of the anions cause a blue shift in the $\lambda_{\text{max}}$ from $\sim600$ nm to near the wild-type value (Table II), consistent with a decrease in the distance of charge separation. This indicates that upon replacement of Asp-85, exogenous anions can substitute for the protein counterion, presumably by binding to the PSB and/or Arg-82. An electrostatic interaction of anions with the PSB in D85A and D85N is further supported by the observed increase in the SB $pK_a$ by 0.3–0.5 units in the presence of 2 M NaCl (Table III). The fact that relatively high anion concentrations are required to induce changes in the $\lambda_{\text{max}}$ as well as in the SB $pK_a$ indicates that the PSB in these mutants has a limited accessibility to aqueous solutions. This interpretation is consistent with a previous study that measured the reactivity of the Schiff base of mutants in the dark, using hydroxyamine as a probe. D85A and D85N revealed a 30-fold reduction in the rate of reaction, compared with eBR, whereas D85E showed a 10-fold increase (35). This difference in the accessibility of the PSB could explain why carboxylic acids generally cause a spectral shift in D85E but not in the D85A and D85N mutants. Significant changes in the absorption maximum are also observed for neutral substitutions of Asp-212 in the presence of salts (Table II). However, the $\lambda_{\text{max}}$ values are generally blue shifted relative to the wild type, suggesting that the solute anions do not replace the endogenous counterion. It is possible that some of the larger anions that shift the $\lambda_{\text{max}}$, of these mutants to near 500 nm induce a structural perturbation in the vicinity of the Schiff base. In D212A and D212N large spectral changes occur even at low anion concentrations. An increased accessibility of the Schiff base to water in these mutants is also suggested by the light instability (6, 26) and the enhanced reactivity with hydroxyamine (35). For R82Q and D212E minor variations in the $\lambda_{\text{max}}$, are observed in the various anion solutions, consistent with the limited accessibility of the Schiff base to aqueous agents (35). The mutants R82Q and D85E are known to display a purple to blue transition near neutral pH (8, 9), and it is likely that the $pK_a$ of this transition is altered in the presence of different salts.

Studies by Blatz et al. (36) have shown that the absorption maxima of protonated Schiff bases of retinal in nonpolar solvents undergo a progressive red shift, as the ionic radius of the halide increases. The mutants R82Q, D85A, D85E, D212A, D212N, and D85N/D212N all display significant variations of the $\lambda_{\text{max}}$ in the presence of halides (Fig. 8). However, a red shift in the $\lambda_{\text{max}}$ with increasing ionic radius in the order $F^- < Cl^- < Br^- < I^-$ is only observed in the case of R82Q and D85E. There are several possible explanations for the lack of correlation in our experiments. First, aqueous solutions are polar solvents, and hydrogen bonding to the SB imine is not prevented. Second, mutations of the Schiff base environment can affect the ability of anions to interact with the PSB to different extents. Third, the opsin shift in bR results from interactions with the polyene chain, in addition to those involving the PSB (37–39). The above mutations could affect both types of interactions. Fourth, resonance structures with partial positive charges located for example on retinylidene C-15, C-13, or C-5 may become stabilized in the mutants. Based on the magnitude of the anion-induced spectral shifts in these mutants, it is evident that the interactions between the PSB and its counterions play an important role in regulating the wavelength of absorbance in bR.

In the double mutant D85N/D212N formation of a chromophore is restored in the presence of various salts. Thus, in the absence of a protein counterion in this mutant, solution anions can substitute and promote Schiff base protonation. This effect apparently is due to a direct electrostatic interaction between anions and the Schiff base. In support of this idea, the $pK_a$ of the SB is increased at higher anion concentrations (Fig. 7), and the absorption maximum of the mutant is sensitive to the type of solution anion present (Table II). The spectral properties of the D85N/D212N double mutant in the presence of salt are strikingly similar to those of the so-called acid-purple form of wild-type bR (28, 40–42). This conclusion is supported by (i) the color, (ii) the effect of anions on the $\lambda_{\text{max}}$, (iii) the stability of the purple chromophore at low pH, (iv) the isomer composition of the retinal chromophore, and (v) photocycle measurements. The acid-purple state of bR arises from the acid-blue state by lowering the pH below 1 with hydrochloric acid or by selective binding of anions at pH $<2$ (28, 40, 42). Based on studies with mutants (9), it appears that Asp-85 is protonated in the blue form of bR ($pK_a$ of protonation $\sim3.5$ and 2.9 in micelles and purple membrane, respectively; cf. Refs. 23 and 40), and at pH 1 Asp-212 is presumably protonated as well. Indeed, recent Fourier transform infrared measurements suggest that one carboxylate group, possibly Asp-212, becomes protonated during the chloride-induced transition from the blue to acid-purple (42). It is therefore likely that in the acid-purple form exogenous anions replace the intrinsic counterion. This proposal is corroborated by the experiments with wild-type eBr and mutant proteins in DMPC/CHAPS/SDS mixed micelles at low pH. In the presence of various salts unfolding of the protein, normally occurring near pH 3, is not observed (Fig. 10). The variation in the $\lambda_{\text{max}}$ seen for eBr and the D85N mutant with the same anion at pH 1.9 could reflect differences in the $pK_a$ of Asp-212 and in the nature of the side chain at position 85 (protonated aspartate versus asparagine). These studies demonstrate that solution anions function as counterions to the PSB and stabilize the folded protein, when both Asp-85 and Asp-212 are neutralized by mutation and/or by protonation at low pH.

It is instructive to compare the Schiff base counterion environment of eBr with that of other retinal-based pigments. In halorhodopsin, which functions as a light-driven chloride pump in Halobacterium halobium, the residues corresponding to Asp-212 and Arg-82 are conserved, whereas Asp-85 is replaced by the neutral Thr (residue 111). This substitution could be responsible for the increased opsin shift ($\lambda_{\text{max}}$, at 578 nm relative to bR) and a weakening of the electrostatic interaction between the PSB and its counterion, as suggested by resonance Raman spectroscopy (43). Besides, several properties of halorhodopsin, namely the SB $pK_a$ of 7.4, the increased SB $pK_a$ in the presence of salts, and the anion-induced blue shift of the $\lambda_{\text{max}}$, (44), are highly similar to those observed for the D85A and D85N mutations in bR. A close relationship between the two halorhodopsin is underscored by recent studies which indicate that the acid-purple form of bR is capable of translocating halides (45). Based on this analogy it is plausible that anions interact directly with the PSB also in halorhodopsin. In the case of bovine rhodopsin, Glu-113 in transmembrane helix C has been identified as the Schiff base counterion by site-specific mutagenesis (46, 47). Its substitution by neutral amino acid residues causes a large reduction in the $pK_a$ of the SB to around 6. In the presence of soluble anions formation of a PSB is promoted in these mutants and the $\lambda_{\text{max}}$ shows anion-dependent shifts (46, 47), analogous to the properties of D85A and D85N in bR.

In summary, our results indicate that Asp-85 serves as the

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1. H. Otto, T. Marti, H. G. Khorana, and M. P. Heyn, unpublished observations.
retinylidene Schiff base counterion in the ground state of bR. During the photocycle Asp-212 may transiently function as a counterion to the chromophore, for example when Asp-85 is protonated. Further studies are needed to identify the Schiff base counterion at the intermediate states of the photocycle and to evaluate the precise roles of Asp-212 and Arg-82 in the proton translocation process.

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