Retinal Isomerization in Bacteriorhodopsin Is Controlled by Specific Chromophore−Protein Interactions. A Study with Noncovalent Artificial Pigments†

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ABSTRACT: It has previously been shown that, in mutants lacking the Lys-216 residue, protonated Schiff bases of retinal occupy noncovalently the bacteriorhodopsin (bR) binding site. Moreover, the retinal−Lys-216 covalent bond is not a prerequisite for initiating the photochemical and proton pump activity of the pigment. In the present work, various Schiff bases of aromatic polyene chromophores were incubated with bacterioopsin to give noncovalent pigments that retain the Lys-216 residue in the binding site. It was observed that the pigment's absorption was considerably red-shifted relative to the corresponding protonated Schiff bases (PSB) in solution and was sensitive to Schiff base linkage substitution. Their PSB pK_a is considerably elevated, similarly to those of related covalently bound pigments. However, the characteristic low-pH purple to blue transition is not observed, but rather a chromophore release from the binding site takes place that is characterized by a pK_a of ∼6 (sensitive to the specific complex). It is suggested that, in variance with native bR, in these complexes Asp-85 is protonated and Asp-212 serves as the sole negatively charged counterion. In contrast to the bound analogues, no photocycle could be detected. It is suggested that a specific retinal−protein geometrical arrangement in the binding site is a prerequisite for achieving the selective retinal photoisomerization.

Bacteriorhodopsin (bR)[1] is the integral protein of the purple membrane of *Halobacterium salinarum* and serves as a light-driven proton pump (for recent reviews, see refs *1*−*3*). The protein is composed of seven transmembrane helices enclosing the binding pocket for an *all-trans*-retinal chromophore, which is bound covalently to Lys-216 via a PSB. Absorption of a photon by the retinal eventually induces isomerization around the C_{13}═C_{14} double bond (*4*), initiating a photocycle with several distinct spectroscopic intermediates, J_{625}, K_{590}, L_{550}, M_{412}, N_{560}, and O_{640}.

The necessity of the retinal−Lys-216 covalent bond for normal pigment function has been addressed previously using K216A and K216G bR mutants, in which the retinal cannot be covalently bound (*5*, *6*). The mutants were reconstituted with retinal alkylamine Schiff bases, thus mimicking the wild type in a way that the binding site includes a retinal PSB but lacks a covalent bond to the protein. The location of the PSB in the binding site in these "noncovalent" (NC) pigments was probably similar to native bR, since Lys-216 was replaced by a smaller residue creating space for the alkyl side chain that mimicked the lysine residue. Interestingly, it was shown that both NC bR pigments (K216G and K216A) exhibit normal photocycle and pumping activity. It was concluded that the covalent bond between the retinal chromophore and the protein backbone is not a prerequisite for the basic structure and for the characteristic photochemical features of bR, including its proton pump activity. In a similar fashion, analogous NC pigments of visual pigments were prepared by incorporating 11-*cis*-retinal Schiff bases into the binding site of two rhodopsin mutants, K236G and K296A. Similarly to bR, it was shown that the covalent linkage between the retinal and the opsin is not required for activation of the visual cascade (*7*).

The fact that the covalent bond to Lys-216 is not a prerequisite for the selective photoisomerization around the C_{13}═C_{14} double bond of bR is mechanistically relevant. Thus, it is evident that the conformational changes in Lys-216 following light absorption, as detected by FTIR studies (*8*, *9*), do not control the specificity of retinal isomerization in bR. It was suggested that isomerization occurs selectively around the C_{13}═C_{14} double bond due to excited-state stabilization of the positive charge around C_{13} by Asp-85 and Asp-212 residues (*10*). This explanation takes into account the electrostatic interactions of the PSB in the protein binding site. It was suggested that the rate of isomerization in D85N and D212N bR mutants was significantly reduced due to reduced stabilization of the PSB by these residues.

In this work, we address the question as to whether noncovalently bound PSB pigments (NC) can be prepared in native bacterioopsin that retains its Lys-216 residue in its

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[1] Abbreviations: bR, bacteriorhodopsin; SB, Schiff base; PSB, protonated Schiff base; NC, noncovalently bound PSB pigments; CB, covalently bound pigments; CD, circular dichroism.

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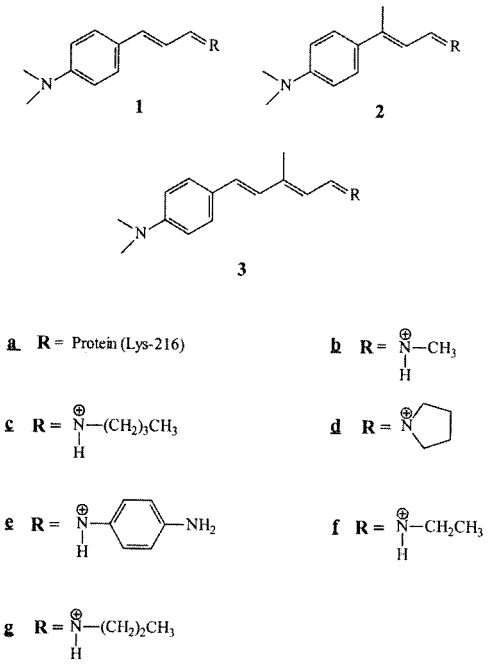
Scheme 1



binding site. Furthermore, we have examined the possibility that the specific location of such PSBs in the binding site plays a significant role in controlling the photocycle activity and retinal isomerization in bR. Accordingly, we have prepared NC pigments by reconstituting the native apomembrane with Schiff bases of aromatic chromophores. We observed that, similarly to the corresponding pigments that are covalently bound to Lys-216, the absorption of these nonbound pigments is considerably red-shifted relative to that in an EtOH solution. Moreover, their PSB pK_a is considerably elevated. Nevertheless, we show that in such NC pigments Asp-85 is protonated at neutral pH so that the blue to purple transition does not take place upon lowering the pH. However, in variance with the bound analogues (CB), no photocycle could be detected. It is suggested that a specific retinal−protein geometrical arrangement in the binding site is a prerequisite for achieving the selective retinal photoisomerization that drives the photocycle.

MATERIALS AND METHODS

Apo-Membrane Preparation. Apo-membranes were prepared from bR and its D85N and R82Q mutants by irradiation in the presence of 1 M hydroxylamine. The apomembrane was washed 4 times to eliminate hydroxylamine contamination.

Aromatic Schiff Base Preparation. The retinal analogues were synthesized according to previously described methods (*11*). Condensation of aromatic chromophores **1**−**3** (Scheme 1) with excess of the appropriate amine was carried out in EtOH at room temperature for 30 min. The formation of the Schiff base was confirmed by monitoring the blue-shifted absorption and its complete transition, following acidification with trichloroacetic acid, to red-shifted PSB.

Noncovalent Aromatic Pigment Preparation. Apo-membrane protein was incubated with an equimolar amount of the appropriate Schiff base for 30 s. The absorption spectra were monitored following the addition of the Schiff base until full reconstitution was observed. Unless otherwise stated, reconstitution was carried out at pH 7 using 5 mM phosphate buffer.

Determination of pK_a Values. The apparent pK_a values of the Schiff base in solution were measured in a 50% EtOH/water solution containing 10 mM of the appropriate phosphate buffer. To determine the pK_a of NC pigments, aliquots of 50 mM sodium hydroxide were added to freshly prepared wt or mutant pigments at pH 9 in the presence of 5 mM Borax buffer and 100 mM sodium chloride (titration at low salt concentrations was carried out without sodium chloride). Determinations of pK_a below 7 were carried out by adding 50 mM hydrochloric acid to freshly prepared pigment at pH 7.8 in the presence of 5 mM phosphate buffer and 100 mM sodium chloride (titration at low salt concentrations was without sodium chloride). The absorption spectra were recorded immediately following pigment formation and the addition of base or acid to minimize the hydrolysis process. For each point along the titration curve, a freshly prepared NC pigment was used. All titrations were carried out at room temperature. The titration data were fitted to the function (derived from the Henderson−Hasselbach equation): $\Delta A = \Delta A_{max}/[1 + 10^{n(pK_a - pH)}]$, in which the parameters are ΔA and ΔA_{max}, the absorbance difference and maximum absorbance difference, respectively, between the two states; n, the number of protons participating in the above transition; and pK_a, the midpoint of the titration.

Pulsed Laser Photolysis. Pulsed laser photolysis was carried out as previously described (*12*) using a Nd:YAG laser system, monitoring with a continuous 75 W Xe source. Signals were recorded using a photomultiplier and a 2440 Tektronix digital oscilloscope. Photolytic effects due to the monitoring beam were minimized by placing an interference filter between the lamp and the sample and by using a mechanical shutter synchronized with the laser pulse. Data were averaged and analyzed on a PC. Due to instability of the pigment, the monitoring light (no laser) reference (I_0) was recorded separately after each (laser on) point (I), and averaging was carried out sequentially for the collected set of (100−1000) I/I_0 values. Freshly prepared wt NC pigments were monitored at pH 7.5 in the presence of 10 mM phosphate buffer. R82Q NC pigments were prepared at pH 9 in the presence of 10 mM Borax buffer. All samples were replaced after each set of experiments.

Low-Temperature Photochemistry. Freshly prepared NC pigments were prepared in 66% (v/v) glycerol solution. The samples were cooled gradually using liquid nitrogen in a transparent quartz cryostat. Spectra were recorded, starting at −160 °C and subsequently at 30 °C intervals up to −30 °C before and after illumination at each temperature, with a 570 nm cutoff filter for NC pigments derived from chromophore **3**. Pigments derived from chromophores **1** and **2** were illuminated with a 470 nm cutoff filter. All illumination experiments were carried out with a halogen lamp with an output of 150 W equipped with a heat-absorbing filter.

Spectroscopic Measurements. Absorption spectra were recorded on an HP 8450A diode array spectrophotometer. CD measurements were recorded on an AVIV circular dichroism spectrometer, model 202.

RESULTS

Generation of the NC Pigments. Incubation of bacterioopsin with several Schiff bases of aromatic retinal analogues

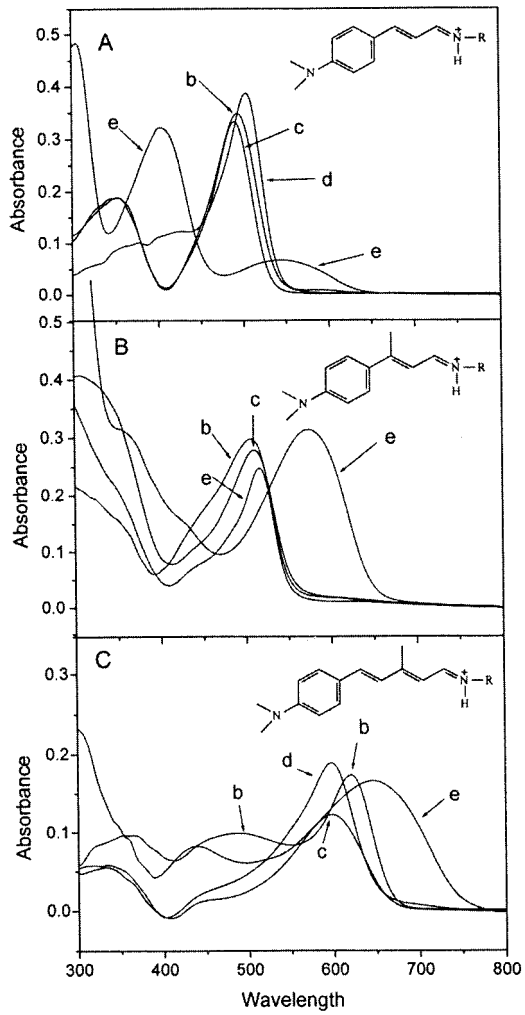


FIGURE 1: Absorption spectra of NC pigments derived from (A) Schiff bases **1b**−**e**, (B) **2b**−**e**, and (C) **3b**−**e**.

(Scheme 1, **1b**−**e**, **2b**−**e**, and **3b**−**e**) led to the prompt formation of a red-shifted absorption characteristic of protein-embedded PSB (Figure 1). Identification of such PSBs as "NC" (unbound) pigments stems from their characteristic absorption, differing from that of the respective covalently bound (CB) pigments obtained by reconstitution of the apo-membrane with the corresponding aldehydes (see below). The fast formation of the NC pigments relative to the respective bound pigments supports other evidence indicating that chromophore penetration into the apo-membrane binding site is not the rate-limiting step in the reconstitution of bR (*13*). The NC pigments were stable for about 10 min (at room temperature) before hydrolysis occurred, followed by formation of the corresponding covalently bound pigment (Scheme 1, **1a**, **2a,** and **3a**).

The absorption maxima of the NC pigments (**3b**−**d**) are red-shifted relative to the CB pigment (**3a**) and relative to the PSB absorption maxima in EtOH (Table 1, Figure 1C). The absorption maxima of the NC pigments (**1b**−**d**, **2b**−**d**), characterized by a shorter polyene chain, are slightly blue-shifted relative to the CB (**1a**, **2a**) and red-shifted relative to the PSB in EtOH (Table 1, Figure 1A,B). These results indicate that the PSB environment in the binding site is similar in both the NC and CB aromatic pigments but differs significantly from that prevailing in EtOH solution. The absorption maxima of NC pigments **1e**, **2e**, and **3e**, in which the aromatic chromophore is condensed with aminoaniline (Scheme 1), are strongly red-shifted relative to pigments **1a**, **2a**, and **3a**. The effect is attributed to a large π-electron delocalization, demonstrating the feasibility of designing NC pigments with a highly red-shifted absorption. Control experiments with native bR have shown that addition of the above Schiff bases to bR at pH 7 did not form red-shifted absorbing pigments. This is in keeping with the basic assumption that all unbound Schiff bases reside in the retinal binding site. Minor blue-shifts in the absorption maxima relative to EtOH solutions were detected, probably due to adsorption of such unbound Schiff bases to the membrane (data not shown).

CD Spectroscopy. CD spectra of all the bacterioopsin−Schiff base complexes indicated that the PSBs occupy the apo-membrane binding site, substantially displacing the retinal oxime. This conclusion stems from the decrease of the retinal oxime positive band (characterized by a maximum at 360 nm), induced by the reconstitution (Figure 2). Some of the NC pigments exhibited a positive CD peak at the maxima of their absorption (**1b**−**e**, **2d**−**e**, **3b**,**e**, Figure 2). Addition of the respective Schiff bases to native bR (at pH 7) did not indicate a change in the CD spectrum, supporting the conclusion that the NC chromophores are located in the retinal binding site. The CD spectra of the NC-bound pigments **1b** and **3b** were stable at both pH 7 and pH 9, and exhibited a positive band at 510 and 620 nm, correspondingly. However, upon decreasing the pH to 4.5, the positive peak decreased dramatically, indicating that the respective PSBs were released from the binding site (Figure 3A,B).

pK_a of the PSB Linkage in the NC Pigments. The apparent pK_a of the PSB in the NC pigments (**1b**, **2b**, and **3b**) is elevated by about 4 units relative to the apparent pK_a of the same PSB in a 50% mixture EtOH/aqueous buffer solution (Table 2). In the cases of **1b**, **2b**, and **3b**, the pK_a values in 100 mM NaCl are between 10.5 and 11 (Figures 4 and 5; Table 2), and decreased by about 1 pK_a unit in the presence of 1 M NaCl (data not shown). The elevated pK_a found in the presence of high salt concentration (relative to solution)

Table 1: Absorption Maxima Values for the CB Pigments, the NC Aromatic Pigments, and the Corresponding PSB in Solution

	1			**2**			**3**		
	PSB[a]	pigment[b]	OS (cm^{-1})[c]	PSB[a]	pigment[b]	OS (cm^{-1})[c]	PSB[a]	pigment[b]	OS (cm^{-1})[c]
a	460	508	2050	460	520	2500	510	578	2300
b	460	492	1400	460	514	2300	510	618	3430
c	460	494	1500	460	510	2130	510	600	2950
d	472	504	1350	476	516	1650	526	600	2350
e	532	545	450	542	574	1030	588	650	1620

[a] Absorption maxima (nm) in ethanol solution. [b] Absorption maximum (nm) of the pigment at pH 7. [c] Opsin shift (OS) is defined as the difference in energy between the absorption of the chromophore PSB in ethanol solution and that of the pigment.

Table 2: pK_a Values for the CB Pigments, the NC Pigments, and the Corresponding PSB in Solution

	pK_a (PSB)				pK_a[d]		
chromophore	solution[a]	CB pigment[b]	NC pigment[c]	NC D85N[c]	NC pigment	NC R82Q	NC D85N
1b	7.1 ± 0.07	12	10.8 ± 0.1	ND	5.7 ± 0.15	6.4 ± 0.1	6.9[e] ± 0.1
2b	6.6 ± 0.1	11.5	10.6 ± 0.1	11.5 ± 0.04	5.4 ± 0.2	ND	ND
3b	6.2 ± 0.06	12	10.9 ± 0.1	10.8 ± 0.04	6.3 ± 0.05	6.3 ± 0.05	6.5[e] ± 0.07

[a] Measured in ethanol/water, 1:1, solution containing 10 mM phosphate buffer. [b] The values are not accurate, due to degradation at high pH values (measured in 0.1 M NaCl). [c] Measured in 0.1 M NaCl with freshly prepared samples. [d] pK_a values for the chromophore release process from the binding site, measured in 0.1 M NaCl. [e] Measured in 5 mM phosphate buffer.

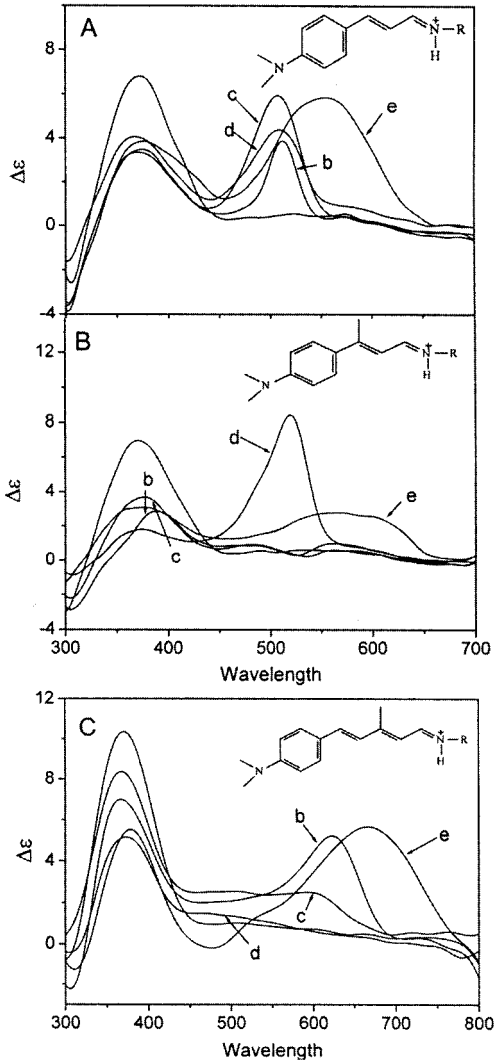


FIGURE 2: CD spectra of bacterioopsin (—) and NC pigments derived from (A) Schiff bases **1b**−**e**, (B) **2b**−**e**, and (C) **3b**−**e**.

indicates that the significant effect of the protein actually involves the PSB environment and is not due to an unusual effect of surface potential, which might distort the measured pK_a due to low pH on the surface relative to the bulk solution. The high pK_a values of the PSB in the NC pigments indicate that the PSB protein environment is considerably different from that prevailing in solution and resembles the environment of the PSB in the CB pigments (**1a**, **2a**, and **3a**). Reconstitution of apo-membrane with Schiff base **3b** leads to a mixture of two pigments absorbing at 620 and ~520 nm (Figure 1C). Upon titration of the PSB, it is clear that both pigments experience a transition to a blue-shifted absorbing Schiff base, indicating a similar pK_a for the two pigments (Figure 5).

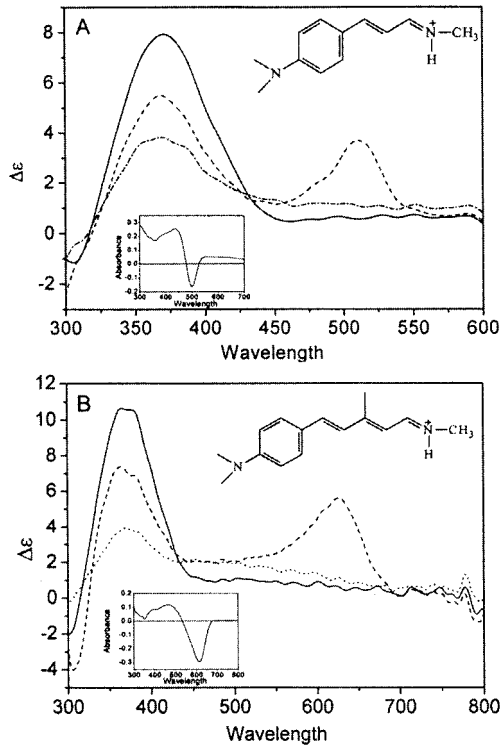


FIGURE 3: CD spectra of NC pigments at different pH values. (A) Bacterioopsin at pH 7 (—), and NC pigments **1b** at pH 7 (- - -) and at pH 4.5 (— · —). Inset: Difference absorption spectrum between pH 4.5 and 7. (B) CD spectra of bacterioopsin at pH 7 (—), and NC pigments **3b** at pH 7 (- - -) and at pH 4.5 (— · —). Inset: Difference absorption spectrum between pH 4.5 and 7.

It was found that upon lowering the pH of the apo-membrane−PSB complex, the chromophore affinity to the binding site decreased dramatically. Rejection from the binding site, which is correlated with the CD results described above, is accompanied by a strong blue shift in the absorption maxima. The titration is reversible, and is attributed to the titration of a protein residue, which in its deprotonated form stabilizes the PSB in the binding site. The pK_a of the transition is 5.7, 5.4, and 6.3 for the NC pigments, **1b**, **2b**, and **3b** (Figures 6 and 7; Table 2), respectively. Upon lowering the pH of the NC **3b** pigment, the absorption at 620 nm is shifted to a broad band around 490 nm, whereas the band at 520 nm did not change (Figure 7). We note that rejection of the PSB from the binding site is in contrast to the behavior of pigments **1a**, **2a**, and **3a**, characterized by a covalent bond to Lys-216. In this case, lowering of the pH induces the characteristic purple to blue transition with no chromophore release.

The Pigment Photocycle. The photochemical activity of the NC pigments **1b**,**c** and **3b**,**c** was investigated with reference to those of the corresponding CB pigments **1a** and

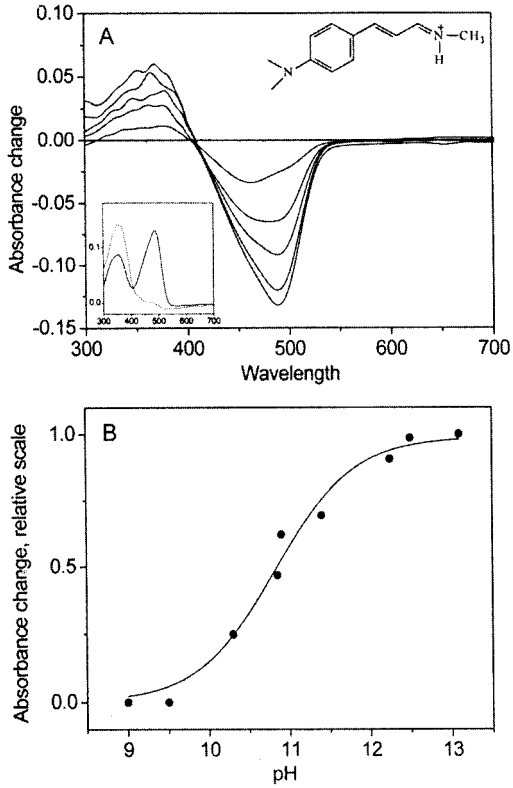


FIGURE 4: Titration of PSB in NC pigment **1b**. (A) Difference absorption spectra at $(pH)_I$ − pH 9. Spectra are shown at pH 10.3, 10.85, 11.4, 12.25, and 12.5. Inset: Absorption spectra of NC pigment **1b** at pH 9 (—) and 12.5 (···). (B) pH dependence of absorption change monitored at 495 nm. The solid line is the best-fit titration with a pK_a of 10.8 ± 0.08 and n = 0.9.

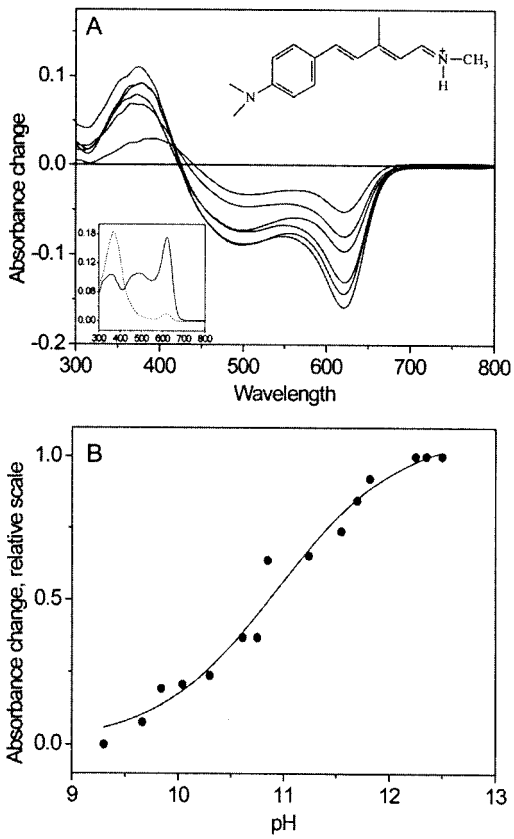


FIGURE 5: Titration of PSB in NC pigment **3b**. (A) Difference absorption spectra at $(pH)_I$ − pH 9. Spectra are shown at pH 9.84, 10.3, 10.85, 11.3, 11.7, and 11.8. Inset: Absorption spectra of NC pigment **3b** at pH 9.3 (—) and 11.8 (···). (B) pH dependence of absorption change monitored at 615 nm. The solid line is the best-fit titration with a pK_a of 10.95 ± 0.1 and n = 0.75.

3a. Neither room-temperature laser flash photolysis nor low-temperature steady-state illumination of the NC pigments led to the detection of any light-induced absorbance changes. A lower limit of $<10^{-3}$ was set for the quantum efficiency of the corresponding photocycles. The lack of detectable, transient or stable, intermediates is at variance with the behavior of pigments **1a** and **3a**, which exhibited a K-like intermediate in the case of **1a** and K- and M-like species for **3a** [in correlation with (*11*)]. This indicates that a suitable protein−chromophore arrangement in the binding site is necessary for initiating the characteristic bR photocycle.

Noncovalent Pigments in D85N and R82Q Mutants. To evaluate the effect of Asp-85 on binding of the Schiff base analogues, we incubated the various Schiff bases with the apo-membrane of mutant D85N. Similarly to native bacterioopsin, the various Schiff bases yielded red-shifted pigments. It was revealed that the absorption maxima of D85N NC pigments **3b**, **3c**, **3f**, and **3g** (Figure 8C; Table 3) are highly sensitive to the nature of the Schiff base side chain. Upon increasing the chain length from two (**3f**) to four carbons (**3c**), the absorption maximum is red-shifted by about 50 nm, from 550 to 600 nm, respectively. This is in contrast to D85N NC pigments **1b**, **2b** and **1c**, **2c** in which a similar increase in side chain length did not significantly affect the absorption maxima (Figure 8A,B; Table 3).

The pK_as of the PSB in D85N NC pigments **2b** and **3b** are similar to those of NC pigments **2b** and **3b** in wt bR [11.5 and 10.8 (Figures 9 and 10) vs 10.6 and 10.95, respectively]. The pK_a of the protein residue, whose protonation destabilizes the chromophore affinity to the binding site in NC pigments **1b** and **3b**, is 6.9 and 6.5 (5 mM buffer), respectively. These values are close to those reported for the corresponding systems in the wt (Table 2), clearly indicating that D85 is not the amino acid which stabilizes the PSB in the binding site. No photocycle activity was detected for the D85N NC pigments, **3b**, **3c**, **3f**, **3g**, and **1b**, following pulsed laser excitation.

The binding efficiency of Schiff bases **3b**−**f** to the apo-membrane of R82Q is highly dependent on the Schiff base alkyl chain length. An efficient binding is observed upon incubation of Schiff base **3b** with the R82Q apo-membrane, whereas increasing the Schiff base alkyl chain by one carbon (**3f**) decreased the binding efficiency by ca. 50%. A further increase in the alkyl chain length (**3g**) abolished the binding almost completely (Figure 11C). In contrast, inclusion of Schiff bases **1b**−**d** and **2b**−**d** with the R82Q apo-membrane indicated that in these cases the Schiff base alkyl chain length did not affect the binding efficiency (Figure 11A,B). CD spectroscopy confirms that the PSB **3b** is indeed located in the R82Q apo-membrane binding site as indicated by a strong decrease in the retinal oxime band at 360 nm (data not shown). The pK_as of the red to blue absorbing pigment associated with a significant decrease of the chromophore affinity to the retinal binding site of **1b** and **3b** in the R82Q apo-membrane are 6.4 and 6.3, respectively. These values are similar to the pK_a obtained for the same PSB in wt apo-membrane (7.1 and 6.3, respectively; 100 mM NaCl). It is concluded that the Arg-82 residue does not have a strong

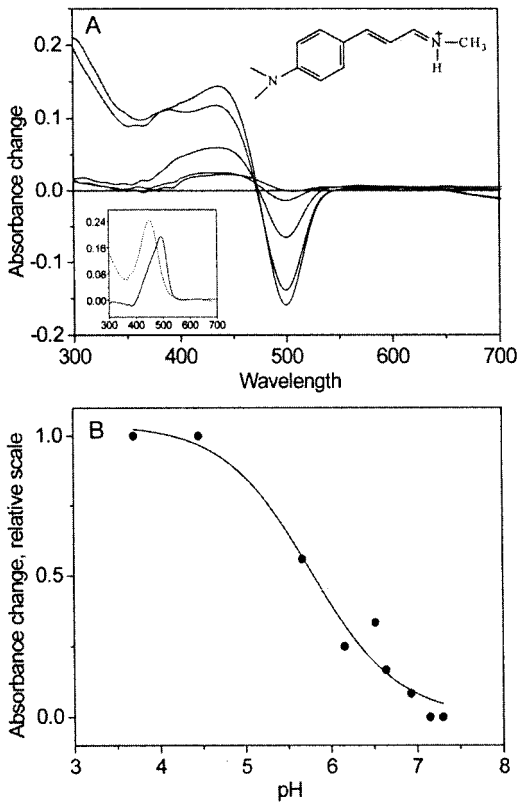


FIGURE 6: Titration of NC pigment **1b** at low pH. (A) Difference absorption spectra at $(pH)_I$ − pH 7.3. Spectra are shown at pH 6.93, 6.64, 5.67, 4.4, and 3.7. Inset: Absorption spectra of NC pigment **1b** at pH 7.3 (—) and 3.7 (···). (B) pH dependence of absorption change monitored at 500 nm. The solid line is the best-fit titration with a pK_a of 5.75 ± 0.15 and n = 0.85.

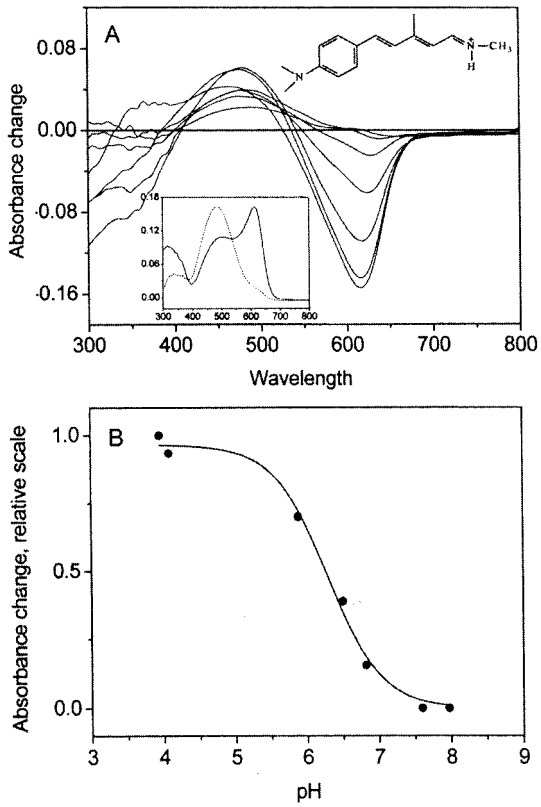


FIGURE 7: Titration of NC pigment **3b** at low pH. (A) Difference absorption spectra at $(pH)_I$ − pH 7.9. Spectra are shown at pH 7.97, 7.6, 6.82, 6.5, 5.88, 4.08, and 3.95. Inset: Absorption spectra of NC pigment **3b** at pH 7.9 (—) and 3.95 (···). (B) pH dependence of absorption change monitored at 615 nm. The solid line is the best-fit titration with a pK_a of 6.28 ± 0.05 and n = 1.15.

interaction with the residues which stabilize the PSB in the binding site.

The photochemical activity of R82Q NC pigments **1b** and **3b** was examined by room-temperature flash laser photolysis and by continuous excitation at low temperatures. In contrast to the corresponding wt NC pigments that did not exhibit any photocycle activity, R82Q NC pigment **3b** exhibits a clear photocycle activity, characterized by formation of an M intermediate (Figure 12B). In addition, the M intermediate was accumulated following irradiation at −40 °C. R82Q NC pigment **1b** did not show any photocycle activity, similarly to wt NC pigment **1b** (Figure 12A). In contrast, R82Q CB pigments **1a** and **3a** exhibited a photocycle in which the M intermediate was clearly observed.

DISCUSSION

It has been previously shown that the retinal−Lys-216 covalent bond is not a precondition for the formation of functional bR pigments (*5*, *6*). Such pigments, prepared by incubating retinal Schiff bases with K216G and K216A bR mutants, exhibit a normal photocycle and pumping activity. In this work, we show that it is possible to form pigments lacking the covalent linkage between Lys-216 and the retinal analogues in native apo-membrane, *while maintaining the native lysine 216 chain in the binding site*. Apo-membranes reconstituted with aromatic Schiff bases **1**, **2**, and **3** (Scheme 1) readily yielded red-shifted absorbing pigments lacking the covalent bond between the PSB and Lys-216. Aromatic Schiff bases derived from chromophores bearing polyene chains longer than two double bonds, as well as retinal alkylamine Schiff bases, did not bind to the binding site. These results show that the presence of Lys-216 in the binding site imposes steric constrains, which reduce the ability of the binding site to accommodate SB analogues longer than two conjugated double bonds. Nevertheless, the observation that the wt binding site can accommodate several alkylamine polyene chains in the presence of Lys-216 is of considerable importance.

The absorption of NC pigment **3b** (Scheme 1) is characterized by two absorption maxima, at 620 and 520 nm (Figures 1C and 5), which can be attributed to two different conformations of the PSB in the binding site. Reconstitution of the D85N and R82Q apo-membrane with Schiff base **3b** results in different 520 nm/620 nm ratios (Figures 8C and 11C), demonstrating that the equilibrium between the two conformations is sensitive to binding site alterations. Similar mixtures of pigments were observed with pigments that were prepared by reconstitution of the K216G apoprotein with retinal alkylamine Schiff bases. The reconstitution in this case led to a mixture of pigments characterized by two absorption maxima, at 566 and 630 nm (*5*).

The retinal oxime displacement by the various Schiff base analogues was monitored by CD spectroscopy. Since specific protein−retinal oxime interactions prevailing in the binding site are reflected in a characteristic CD band at 360 nm (*14*), this methodology can efficiently discern between retinal oxime located inside and outside the binding site. Thus, the Schiff base binding to the apo-membrane is monitored by

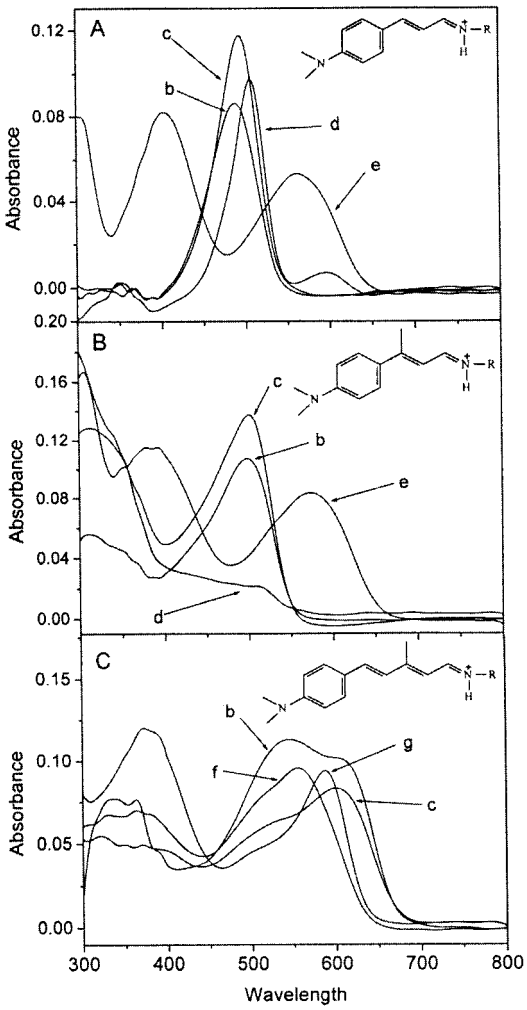


FIGURE 8: Absorption spectra of NC D85N pigments. (A) Absorption spectra of NC pigment derived from Schiff bases **1b**−**e**, (B) **2b**−**e**, and (C) **3b**,**c**,**f**,**g**.

Table 3: Absorption Maxima (λ_{max}, nm) of D85N Aromatic Pigments

	Schiff base		
side chain	**1**	**2**	**3**
b	486	500	512; 610 (shoulder)
f	488	514	556
g	494	512	588
c	494	504	600
d	506	518	598

the subsequent decrease of the retinal oxime elipticity upon its displacement from the binding pocket by the PSB. Part of the NC pigments studied exhibit a clear CD band, providing an additional indication that the PSBs occupy the regular retinal binding site. The efficiency of retinal oxime displacement from the binding site depends on the polyene length, which further supports the observation that the number of conjugated double bonds in the Schiff base is a crucial factor in controlling the binding efficiency.

The large opsin shift (*15*) of the NC pigments is similar to the opsin shift of the CB pigments **1a**, **2a**, and **3a**. A particularly significant red-shifted absorption is observed for NC pigments derived from aromatic Schiff base **3**, exceeding in most cases the opsin shift of the CB pigment **3a** (Table 1). The large opsin shift in native bR is explained mainly

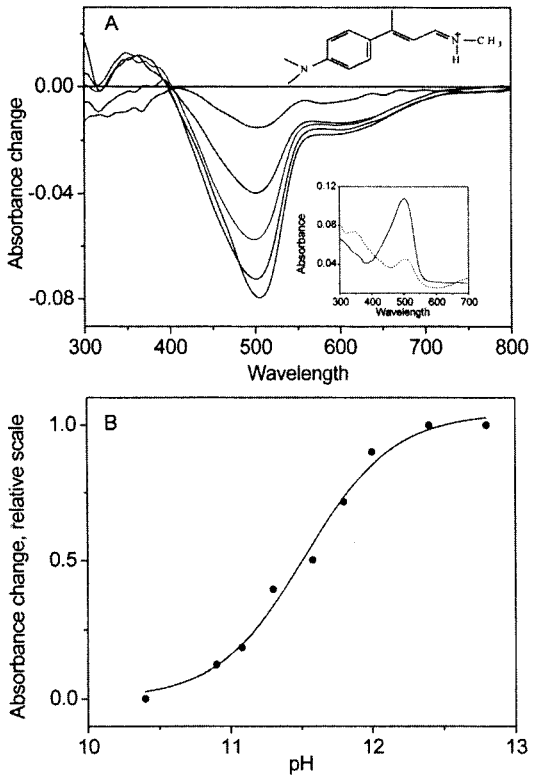


FIGURE 9: Titration of PSB in NC D85N pigment **2b**. (A) Difference absorption spectra at $(pH)_I$ − pH 10.3. Spectra are shown at pH 11.08, 11.58, 11.8, 12, and 12.38. Inset: Absorption spectra of NC D85N pigment **2b** at pH 10.3 (—) and 12.38 (···). (B) pH dependence of absorption change monitored at 502 nm. The solid line is the best-fit titration with a pK_a of 11.5 ± 0.04 and $n = 1.4$.

by a weak PSB counterion interaction relative to ethanol solution (*16*−*18*), and by a ring-chain planarity imposed by the protein binding site (*19*−*21*). In NC pigments, the PSB counterion interaction is probably the major operating factor in inducing the opsin shift, since these aromatic PSBs are already planar in solution. The significant opsin shift detected in the NC pigments suggests that the PSB environments represent weak PSB counterion interaction as observed for the CB aromatic pigments. In native bR, the state of protonation of Asp-85 plays an important role in controlling the absorption maximum of the PSB. Thus, protonation red-shifts the absorption from 568 to 605 nm (*22*, *23*). Similarly, the mutant D85N lacking the negative charge absorbs at 605 nm (*24*). In contrast, Asp-85 mutation does not affect the absorption maxima of the present NC pigments. This may suggest that Asp-85 does not participate in determining the absorption maxima of the NC pigments.

Further evidence for different SB−Asp-85 interactions in the present NC systems, with respect to wt bR, can be derived from the SB pK_a values and their sensitivity to Asp-85 mutation. The high apparent pK_a of the PSB in bR [∼12.2, 150 mM NaCl (*25*, *26*)] relative to solution (∼7.2) was attributed to the protein environment in the vicinity of the PSB. It was suggested that a specific angle between the PSB and D85 allows bound water molecules to form defined structure and to bridge the two groups (*27*). The presence of a bound water molecule was recently confirmed by X-ray diffraction studies (*28*). The apparent pK_a of the PSB in the NC pigments is elevated similarly to the pK_a of the PSB in bR, suggesting a protein environment which considerably

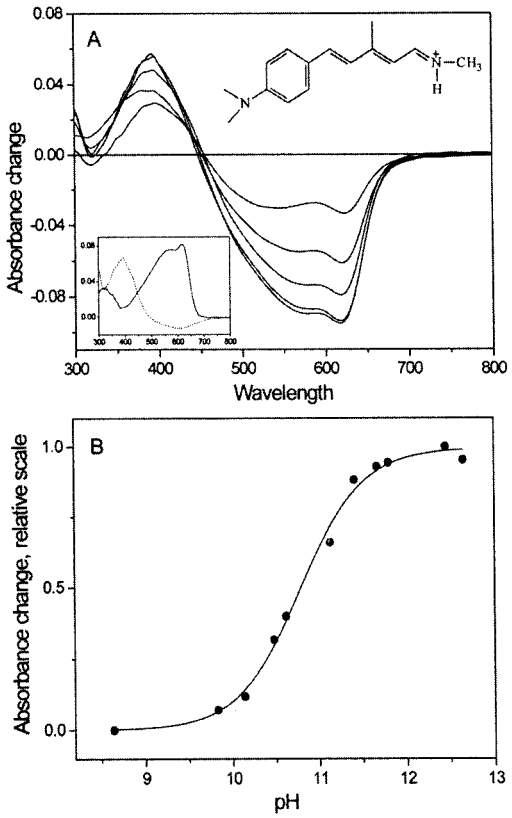


FIGURE 10: Titration of PSB in NC D85N pigment **3b**. (A) Difference absorption spectra at $(pH)_I$ − pH 8.6. Spectra are shown at pH 10.62, 11.13, 11.8, 12.6, and 12.33. Inset: Absorption spectra of NC D85N pigment **3b** at pH 8.6 (—) and 12.33 (···). (B) pH dependence of absorption change monitored at 620 nm. The solid line is the best-fit titration with a pK_a of 10.8 ± 0.04 and n = 1.2.

elevates the apparent pK_a relative to the pK_a in solution. However, despite the similar effects of the protein environments on the pK_a values, the two systems behave differently with respect to Asp-85 mutation. In bR, mutation of Asp-85 decreases the PSB pK_a by ∼5 pK_a units (*24*), while the pK_a of the NC pigment is unaffected by mutation. This result, as well as the insensitivity of the absorption to Asp-85 mutation described above, strongly suggests that *in the NC pigment Asp-85 is protonated, and that another protein residue serves as the counterion to the PSB.* This is consistent with previous observations showing that in artificial bR pigments the pK_a of Asp-85 is highly sensitive to perturbations of the binding site that can substantially elevate its pK_a (*29*).

In bR, the state of protonation of the Asp-85 counterion is best monitored by the purple⇔blue transition at low pH (*22*, *23*). An analogous criterion is not applicable in the case of the counterion that replaces Asp-85 in the NC pigments, due to the instability of these pigments at low pH. Thus, upon lowering the pH, the pigments undergo a dramatic change in their absorption and CD spectra (Figures 3, 6, and 7). The effect is reversible, indicating that Schiff base hydrolysis does not take place. The transition can be readily explained in terms of the titration of an acidic protein residue, which in its protonated form reduces the affinity of the chromophore to the binding site. The resulting displacement of the chromophore induces a blue shift in the absorption maximum. Strong support for the release of the NC PSB from the apo-membrane binding site upon lowering the pH was derived from CD spectroscopy. The CD spectra of the

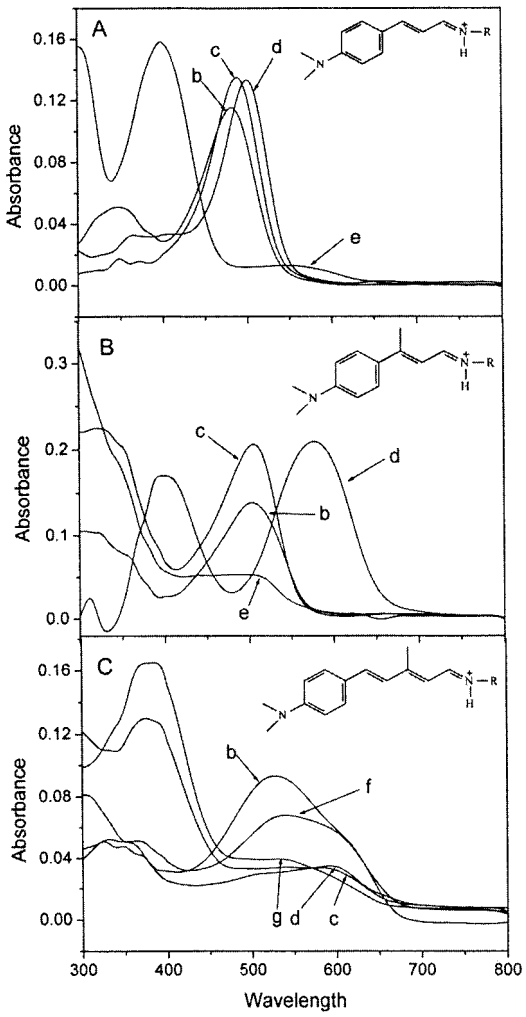


FIGURE 11: Absorption spectra of NC R82Q pigments derived from (A) Schiff bases **1b**−**e**, (B) **2b**−**e**, and (C) **3b**−**d**,**f**,**g**.

NC pigments **1b** and **3b** show a clear CD positive band at 520 and 620 nm, respectively (Figure 3), which disappears at low pH along with the blue shift in the absorption.

We conclude that in the NC pigments, release of the chromophore from the binding site, following protonation of the counterion, replaces the purple→blue transition as the criterion for protonation of the SB counterion. As to the nature of this counterion, we suggest that the negative group, that stabilizes the NC PSB in the binding site is Asp-212, is probably mediated by bound water. This is supported by the observation that reconstitution of D212N apo-membrane with aromatic Schiff base (**1**, **2**, and **3**) did not result in the formation of NC-bound pigments. In native bR, the apparent pK_a of D212 is probably very low (*30*) and may be associated with the acid blue to acid purple transition. In view of our present assignment, in the NC pigments the pK_a of this group is significantly elevated, to about 5 (Table 2). It was shown that in the D85N bR mutant, lowering the pH to ∼5 in the presence of high chloride concentration induces a blue to purple transition attributed to binding of a chloride ion in the vicinity of the PSB (*31*). It is tempting to suggest that in this mutant the pK_a of Asp-212 increases to above ∼5, but can be replaced by chloride as counterion to the Schiff base. It appears, however, that in the present NC pigments, chloride does not bind and cannot replace Asp-212 in stabilizing the PSB.

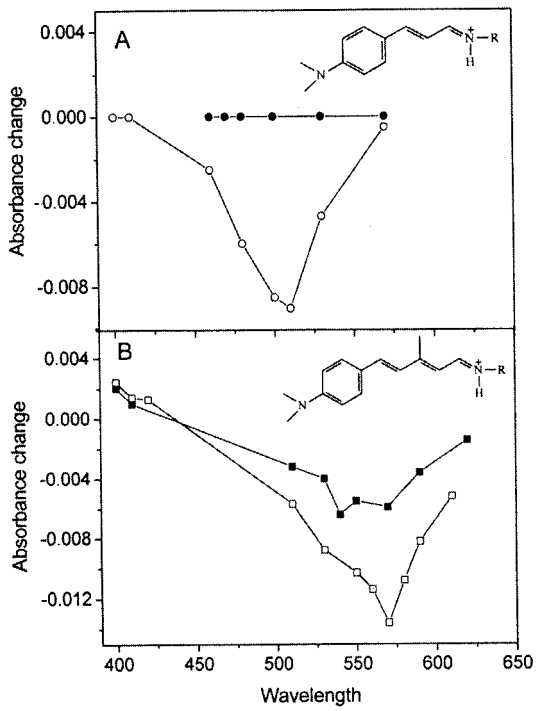


FIGURE 12: Laser-induced transient spectra of NC R82Q pigments after 1 ms. (A) CB R82Q pigment **1a** (○); NC R82Q **1b** (●). (B) CB R82Q pigment 3a (□); NC R82Q **3b** (■).

The low-pK_a transition is similar for both D85N and R82Q NC pigments, demonstrating that (a) Asp-85 and Arg-82 do not participate in the stabilization of the PSB in the binding site and (b) these residues do not interact significantly with the group (Asp-212), which stabilizes the aromatic PSB. This is at variance with the behavior of native bR, where the Arg-82 residue interacts strongly with Asp-85, as evidenced by the strong effect of R82Q mutation on the pK_a of the purple to blue transition (*32*−*34*) and by the X-ray structure of bR (*28*, *35*, *36*). In conclusion, we suggest that in the aromatic NC pigments, the protein adopts a conformation in which Asp-85 is protonated and Asp-212 serves as the PSB counterion. We also propose that in these systems Arg-82 moves toward the extracellular surface and does not interact with the Asp-85 and Asp-212 residues. This structure resembles that of the M photocycle intermediate of bR in which Asp-85 is protonated and Arg-82 moves toward the extracellular side (*35*, *37*, *38*). The protonation of Asp-85 may serve as the trigger for the conformational changes and Arg-82 displacement away from the retinal PSB.

Isomerization of the retinal PSB in bR following light absorption occurs specifically around the C_{13}═C_{14} double bond and is highly efficient (*39*, *40*). Artificial pigments in which the C_{13}═C_{14} double bond was locked by a rigid ring structure did not exhibit any photocycle, most probably implying that they do not experience isomerization around any other double bond along the polyene chain (*41*−*43*). The mechanism by which the protein directs the isomerization to occur specifically around the C_{13}═C_{14} double bond is intriguing and still unclear. Previous work with K216G and K216A pigments (*5*, *6*) as well as our present R82Q NC **3b** data excludes models that attributed the isomerization specificity to conformational changes in the Lys-216 side chain. A plausible explanation was advanced by Song et al. (*10*), who suggested that the isomerization is specific to the C_{13}═C_{14} double bond since the protein stabilizes, in the excited state, a positive charge at C_{13} through interaction with Asp-85 and Asp-212. The question arises concerning the lack of a photocycle in most of the present NC pigments, that may be assigned to their incapability to undergo primary photoisomerization. This is in contrast to the CB pigments **1a** and **3a** for which a clear photocycle activity is observed. Previous studies with NC K216G and K216A mutants, as well as the present NC R82Q **3b** pigment, demonstrate that such incapability to isomerize cannot be ascribed to the lack of the Lys-216 covalent bond between the chromophore and the protein. An alternative approach would be to attribute the effect to the protonated nature of Asp-85 in the NC pigments. However, this would be inconsistent with the presence of photocycles detected for the blue bR membrane (*44*) and its D85N mutant (*45*). We therefore suggest that the NC pigments do not experience isomerization due to steric constrains. The retinal chromophore isomerizes in the binding site only when specific chromophore−protein geometrical arrangements are achieved. Due to steric limitations imposed by the presence of Lys-216 in the native NC pigments, the chromophore cannot adopt a proper conformation (similar to covalent pigments **1a**, **2a,** and **3a**), and a barrier for the isomerization process is imposed.

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