pK_a of the Protonated Schiff Base and Aspartic 85 in the Bacteriorhodopsin Binding Site Is Controlled by a Specific Geometry between the Two Residues[†,‡]

I. Rousso,[§] N. Friedman,[§] M. Sheves,*[,§] and M. Ottolenghi*[,∥]

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel, and Department of Physical Chemistry, The Hebrew University, Jerusalem 91904, Israel

Received January 31, 1995; Revised Manuscript Received June 22, 1995[⊗]

ABSTRACT: The structure and function of the light-driven proton pump bacteriorhodopsin appear to be determined by the exact geometrical conformation of specific groups in the retinal binding site, including bound water molecules. This applies to the pK_a values of the protonated Schiff base, which links the retinal chromophore to Lys[216], and to Asp[85]. In the present work we show that the geometrical constraints imposed by the ring structures of several synthetic retinals can induce substantial changes in the pK_a values of the Schiff base and of Asp[85]. Thus, the artificial pigments derived from 13-demethyl-11,14-epoxyretinal (**2**) and 13-demethyl-9,12-epoxyretinal (**3**) show protonated Schiff base pK_a values of 8.2 ± 0.1 and 9.1 ± 0.1, respectively, as compared with 13.3 in the native (*all-trans*-retinal) pigment. We also suggest that in both systems the pK_a of Asp[85] increases from 3.2 in the native bR to above 9. Analogous, though smaller, effects are obtained for artificial bR pigments derived from 12,14-ethanoretinal (**4**), 11,-13-propanoretinal (**5**), 11,13-ethanoretinal (**6**), and *p*-$(CH_3)_2N-C_6H_4-HC{=}CH-C(CH_3){=}CH-CHO$ **7**. The effects of geometry on the pK_a values (those on Asp[85] being more pronounced) are attributed to the disruption of the original, well-defined, structure in which the Schiff base and its Asp[85] counterion are bridged by bound water molecules. These results are the first to show that it is possible to modify the pK_a values of the Schiff base and Asp[85] in appropriate artificial pigments, without inducing intrinsic pK_a changes in the chromophore or introducing a mutation in the protein. The results bear on the structure of bR and on the mechanisms of its light-driven proton pump in which both Schiff base and Asp[85] moieties play central roles.

The light-induced proton-pumping activity of bacteriorhodopsin (bR)[1] is based on the photocycle of its *all-trans*-retinal protein pigment [for recent reviews, see Mathies et al. (1991), Oesterhelt et al. (1992), Ebrey (1993), and Lanyi (1993)]. The retinal chromophore is bound covalently to the protein via a protonated Schiff base to the ϵ-amino group of Lys[216] (Lewis et al., 1974). Following light absorption, the pigment experiences a series of structural changes associated with several spectroscopically distinguishable intermediates denoted H, I, J, K, L, M, N, and O. The transformation of the L intermediate to M involves Schiff base deprotonation and protonation of D85 (Braiman et al., 1988; Butt et al., 1989; Stern et al., 1989; Gerwert et al., 1990; Metz et al., 1992).

The absence of light-induced Schiff base deprotonation in the native system, i.e., M formation, abolishes the proton-pumping activity, confirming the crucial role of te Schiff base in the biological function of bR. For example, Longstaff and Rando (1987) have shown that methylation of Lys[216] eliminates both the light-induced Schiff base deprotonation and the proton-pumping activity. Thus, understanding the factors that influence the pK_a values of the protonated Schiff base and the acceptor group, D85, is of primary importance. The apparent pK_a of the protonated Schiff base in bR is 13.3 ± 0.3 in water without salts (Druckmann et al., 1982; Sheves et al., 1986), and ~12.2 in 150 mM NaCl (Balashov et al., 1991), while that of D85, as monitored by the transition of the purple pigment to a blue form, is 2.7 (in 0.1 M NaCl) (Jonas & Ebrey, 1991). However, on the basis of the pK_a results of bR mutants, it has been suggested that prior to light absorption by bR the effective ΔpK_a between the donor and the acceptor groups for proton transfer between the Schiff base and D85 is only 5.3−5.7 pH units (Brown et al., 1993). During the photocycle this gap is cancelled by changing the proton affinity of the Schiff base and/or of D85.

The apparent pK_a observed for the protonated retinal Schiff base in bR is unusually high relative to the value of 7.2 observed for model compounds in methanol/water (1:1) solution (Baasov & Sheves, 1986). Several suggestions have been invoked to explain this high pK_a and its possible drop during the photocycle. A polar protein environment in the vicinity of the Schiff base can induce a high pK_a value (Warshel et al., 1984; Sheves et al., 1986; Baasov & Sheves, 1986). During the photocycle, an environmental alteration decreasing the local polarity can occur, being accompanied by a drop in the pK_a value. Another approach attributes this pK_a change to rotation around the 14C−15C retinal single bond (Schulten & Tavan, 1978) or to charge separation

[†] This work was supported by the US−Israel Binational Science Foundation, the Human Frontier for the Promotion of Science, and the Fund for Basic Research (administered by the Israel Academy of Sciences and Humanities).

[‡] This paper is dedicated to Prof. Koji Nakanishi on the occasion of his 70th birthday.

[§] The Weizmann Institute of Science.

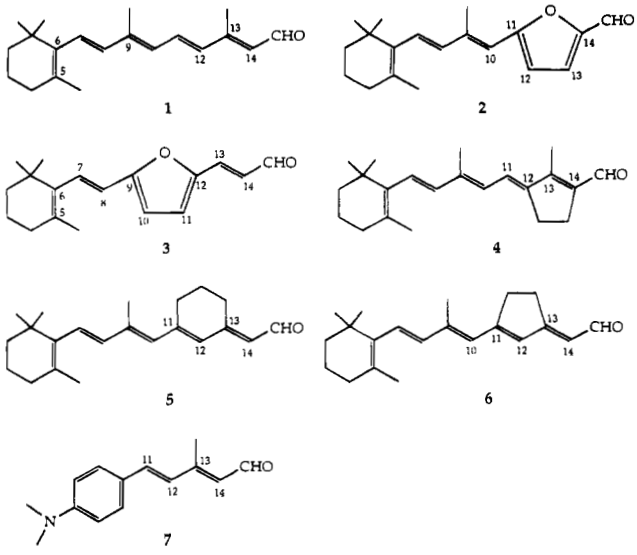
[∥] The Hebrew University.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: bR, bacteriorhodopsin; D85N, a bacteriorhodopsin mutant in which Asp85 was replaced by glutamine; OS, opsin shift; PSB, protonated Schiff base.

0006-2960/95/0434-12059$09.00/0

Chart 1



between the protonated Schiff base and its counterion (Honig et al., 1979). The importance of the relative orientation of donor and acceptor groups for proton transfer was suggested by Scheiner and Hildebrandt (1985) and Scheiner and Duan (1991). Several groups suggested that water molecules are present in the binding site that might stabilize the protonated Schiff base (Du Puis et al., 1980; Hildebrandt & Stockburger, 1984; De Groot et al., 1989). The presence of water molecules in the Schiff base vicinity and their alteration during the photocycle were demonstrated by FTIR studies (Maeda et al., 1992, 1994). We have recently suggested (on the basis of a model compounds study) that a specific angle between the protonated retinal Schiff base and Asp^{85} allows bound water molecules to form a defined structure and to bridge the two groups (Gat & Sheves, 1993). This defined structure, which is associated with stabilizing the donor and acceptor ion pairs, induces a high pK_a for the protonated Schiff base and a relatively low one for Asp^{85}. Light absorption might disrupt the hydrogen network from destabilizing the ion pair and lead to a proton transfer from the protonated Schiff base to Asp^{85}. Recent studies with bR mutants A54V and 49A provided experimental support for this possibility (Brown et al., 1994).

In this study, we approach the question of the role of the binding site geometry, including that of bound water, by studying the pK_a values of the protonated Schiff base and Asp^{85} in artificial bR pigments. In a previous work (Sheves et al., 1986) we have shown that the pK_a of the Schiff base of bR may be modified by inducing intrinsic pK_a changes in the retinal chromophore by its substitution with fluorinated groups. We now demonstrate that pK_a changes in bR may also be induced by geometrical effects. Specifically, it is shown that, by imposing different conformations in the bR binding site by using retinal analogs characterized by different structures in the Schiff base vicinity, the apparent pK_as of both the protonated Schiff base and Asp^{85} are considerably altered. This study reveals that the pK_a of the Asp^{85} residue is more sensitive to conformational changes than that of the protonated retinal Schiff base.

MATERIALS AND METHODS

The retinal analogs were synthesized according to previously described methods: chromophores **2**, **3**, and **7**, after Brock et al. (1983) and Sheves et al. (1985); chromophore **4**, after Fang et al. (1983); and chromophores **5** and **6**, after Sheves et al. (1986). The artificial pigments were prepared by incubating the retinal analogs with the apomembrane at room temperature (Tokunaga et al., 1978) (Chart 1).

Titrations of pigments II−VI were carried out by adjusting the pH with HCl or NaOH in a pigment suspension containing 0.1 M NaCl at room temperature. Pigment VII was titrated with H_2SO_4 without NaCl. The absorption was monitored using a Hewlett Packard 8450A diode array spectrophotometer. The pigment absorption spectra were obtained by subtracting the apomembrane spectrum from the measured spectra.

The pK_a values of the various pigments were determined by a three-parameter function derived from the Henderson−Hasselbach equation:

$$\Delta A = \frac{\Delta A_{max}}{1 + 10^{n(pK_a - pH)}}$$

where the parameters are ΔA and ΔA_{max}, the absorbance difference and the maximum absorbance difference, correspondingly, between the protonated and deprotonated states; n is the number of protons participating in the above transition; and pK_a is the midpoint of titration. Curve fitting was performed using the variable metric Newton−Raphson search algorithm.

RESULTS

To explore the possibility that the specific geometry between the protonated Schiff base and Asp^{85} is crucial for the structure of the binding site (presumably by involving bound water) and thereby affects the apparent pK_a of these groups, we constructed artificial pigments, which are expected to affect the geometry in the binding site. Pigments derived from chromophores **2**−**7** were prepared, and their protonated Schiff base pK_a values were measured along with that of their purple to blue transition, which is due to protonation of Asp^{85} (Table 1). Chromophores **2** and **3** bear a furan ring incorporated into the retinal skeleton, which imposes considerable alterations on the polyene conformation. In bR, the protonated Schiff base titration is associated with an absorption transition from 568 to 460 nm characterized by a pK_a of 13.3 ± 0.3 (Druckmann et al., 1982) and 12.2 ± 0.2 in 0.1 M NaCl. The pigments derived from 13-demethyl-11,14-epoxyretinal (**2**) and 13-demethyl-9,12-epoxyretinal (**3**) exhibit analogous transitions (from 570 to 410 nm), which are associated with the substantially lower pK_a values of 8.2 ± 0.1 and 9.1 ± 0.1, respectively (Figures 1 and 2). The question arises as to whether this considerable decrease in the pK_a values relative to bR is associated with specific chromophore−protein interactions, namely, with a conformational change in the binding site with respect to native bR or with an intrinsic pK_a alteration of the chromophore. To address this question, we compared the pK_as of the two pigments with those of the protonated Schiff bases derived from chromophores **1**, **2**, and **3** in solution (water/methanol, 1:1). The results (Table 1) reveal that the differences in the pK_a values between pigments II and III and their corresponding protonated Schiff bases in solution are 2.2 and 2.4 pK_a units relative to ca. 5 units in bR. We thus conclude that protein−chromophore interactions in II

Table 1: Absorption and pK_a Values for the Artificial Pigments and the Corresponding Chromophores in Solution

	λ_{max} (nm)			pK_a (PSB)			
chromophore/pigment	PSB[a]	pigment (pH 7)	OS[c] (cm^{-1})	solution[d]	pigment[e]	ΔpK_a[f]	pK_a (D85)[e]
1/I	440	568	5100	7.2	12.2 ± 0.2	5.0 ± 0.2	2.7
2/II	436	568[b]	5300	6.0	8.2 ± 0.1	2.2 ± 0.1	≥9
3/III	435	570[b]	5400	6.7	9.1 ± 0.1	2.4 ± 0.1	≥9
4/IV	464	574[b]	4250	7.3	11.5 ± 0.2	4.2 ± 0.2	7.9 ± 0.1
5/V	436	556	5000	7.2	12.1 ± 0.2	4.9 ± 0.2	4.0 ± 0.1
6/VI	438	522	3780	7.3	12.0 ± 0.2	4.7 ± 0.2	3.4 ± 0.1
7/VII	511	580	2300	7.0	12.0 ± 0.2	5.0 ± 0.2	5.2 ± 0.1[g]

[a] Absorption in methanol solution. [b] Absorption of the pigment blue membrane form. [c] Opsin shift (OS) is defined as the difference in energy between the absorption of chromophore protonated Schiff base and the absorption of the pigment. [d] Measured in methanol/water, 1:1, solution. [e] Measured in 0.1 M NaCl. [f] Difference between pigment and solution values. [g] Measured by titration with H_2SO_4 without NaCl.

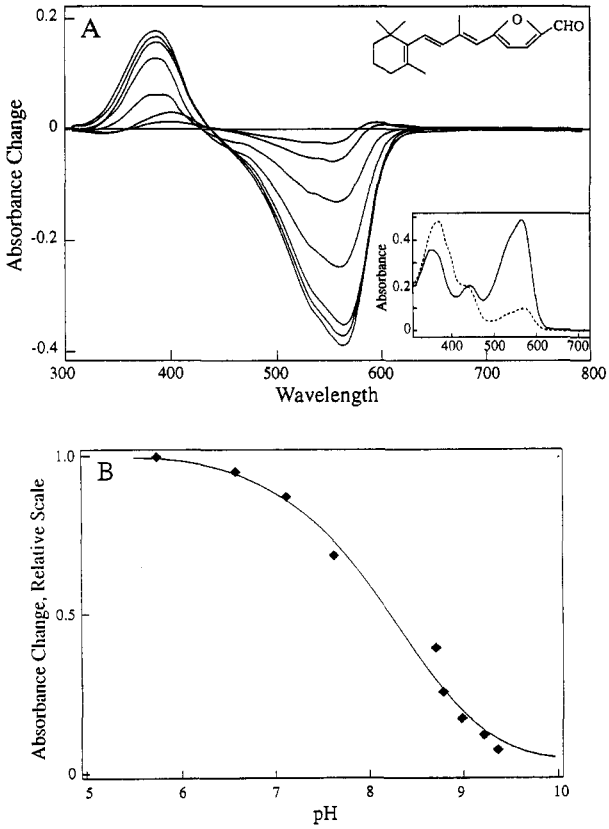


FIGURE 1: Titration of protonated Schiff base in pigment II. (A) Difference absorption spectra at $(pH)_i$ − pH 5.72. Spectra are shown for pH 6.57, 7.12, 7.64, 8.73, 8.81, 9.08, and 9.24. Inset: Absorption spectra of pigment II at pH 5.72 (—) and 9.24 (- - -). (B) pH dependence of absorption change monitored at 570 nm. The solid line is the best-fit titration with a pK_a of 8.2 ± 0.1 and $n = 0.8$.

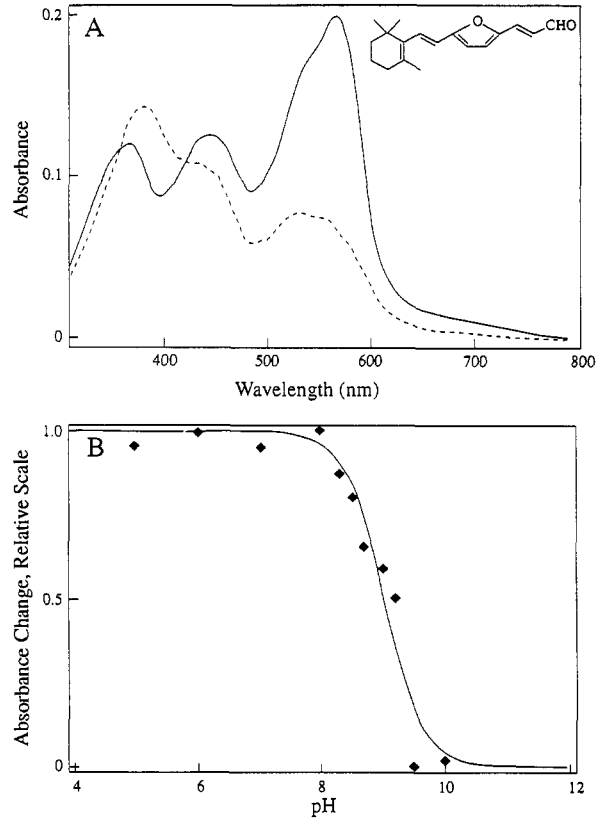


FIGURE 2: Titration of the protonated Schiff base of pigment III. (A) Absorption spectra at pH 6.1 (—) and pH 10 (- - -). (B) pH dependence of absorption change monitored at 570 nm. The solid line is the best-fit titration with a pK_a of 9.1 ± 0.1 and $n = 1.4$.

and III, which are absent in the native pigment, are responsible for a pK_a reduction of ca. 2.5 units with respect to bR.

Bacteriorhodopsin experiences a red shift in its absorption (to 605 nm) with a pK_a of 3.2 (in distilled water). It is currently believed that this transformation is associated with the titration of Asp^{85} [Metz et al., 1992; see Ebrey (1993) and Lanyi (1993) for reviews]. This assumption is also supported by the mutant D85N, which exhibits an absorption (at pH 7) similar to that of the blue membrane, attributed to the absence of the negatively charged Asp^{85} in the binding site. In variance with the behavior of the native system and most artificial pigments (Friedman et al., 1989; Ottolenghi & Sheves, 1989), in both artificial pigments II and III, we did not detect any absorption change in the 1−9 pH range attributable to the purple to blue transition. This observation might indicate that Asp^{85} is protonated in this pH range. To further support this possibility, we prepared artificial pigments of the bacteriorhodopsin mutant D85N derived from chromophores **2** and **3**. In contrast to the behavior of the D85N mutant carrying an *all-trans*-retinal chromophore, the absorption maxima of these two pigments (at pH 7) were very similar to those of pigments II and III, with no detectable red shift (Figure 3). This observation supports the suggestion that Asp^{85} is protonated in the two pigments.

At pH 7, the artificial pigment IV derived from chromophore **4** absorbs at 574 nm. This absorption does not show the characteristic red shift to ca. 600 nm (blue membrane) observed in native bR by lowering the pH to 2.1. However, it undergoes a blue shift to 550 nm by raising the pH above 7. This transition is characterized by a pK_a of 7.9 ± 0.1 (Figure 4). This observation, in combination with the lack of absorption changes at low pH, suggests that the

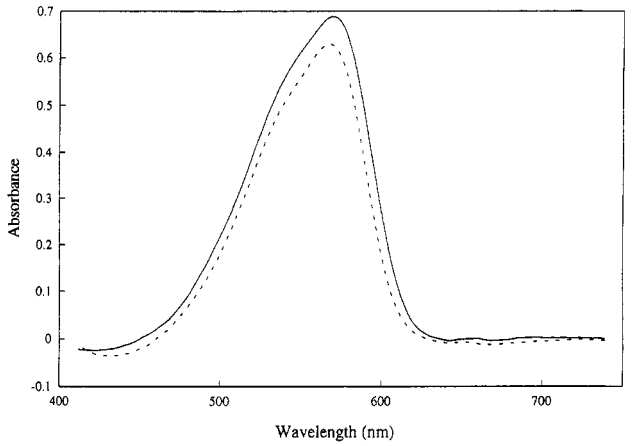


FIGURE 3: Absorption spectra of chromophores **2** (- - -) and **3** (—) incubated with the apomembrane of mutant D85N. Absorption maxima at 567 and 570 nm, correspondingly.

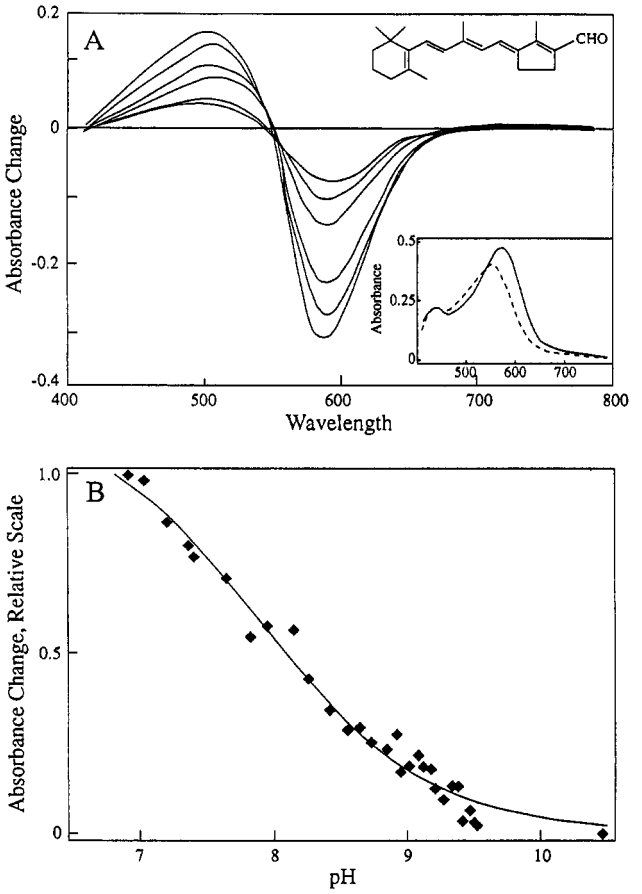


FIGURE 4: Titration of the blue to purple transition of pigment IV. (A) Difference absorption spectra at $(pH)_i$ − pH 7.1. Spectra are shown for pH 7.25, 7.4, 7.6, 7.94, 8.6, 9.0, and 9.5. Inset: Absorption spectra at pH 7.1 (—) and pH 9.5 (- - -). (B) pH dependence of absorption change monitored at 570 nm. The solid line represents the best-fit titration with a pK_a of 7.9 ± 0.1.

pK_a of 7.9 is associated with the titration of Asp^{85}. To check this possibility, we incubated chromophore **4** with the apomembrane of the D85N mutant. As described above, as judged by its absorption, the binding site of this mutant resembles that of blue bR in which Asp^{85} is protonated. The pigment derived from chromophore **4** and the D85N mutant absorbed at 578 nm, similarly to the acid form of pigment IV. Moreover, this absorption did not change following pH elevation to 8.5, in contrast to pigment IV, which experiences

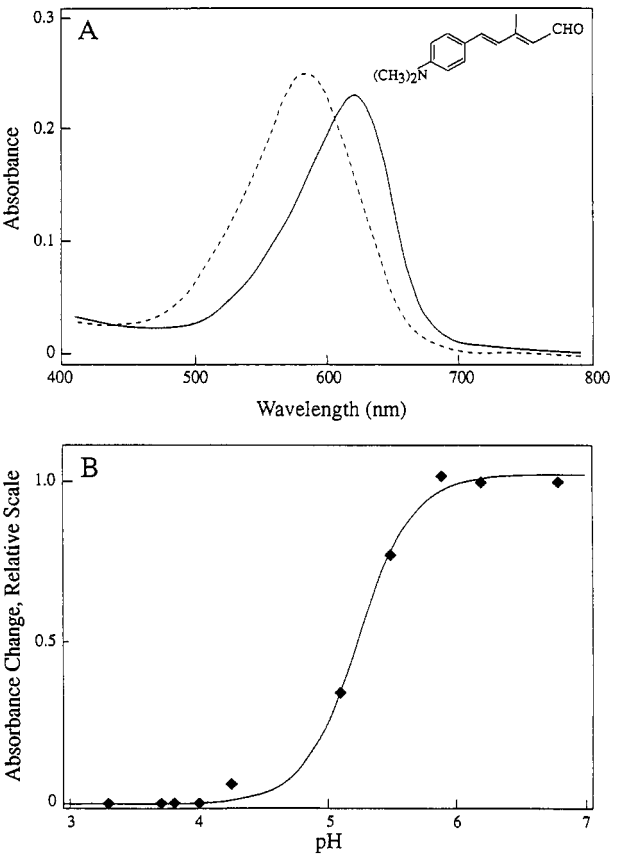


FIGURE 5: Titration of the "purple to blue" transition of pigment VII. (A) Absorption spectra at pH 7 (- - -) and pH 4 (—). (B) pH dependence of absorption change monitored at 620 nm. The titration was carried out in water by adding a 0.1 N solution of H_2SO_4. The solid line represents the best titration fit with a pK_a of 5.2 ± 0.1.

a blue shift to 550 nm, supporting the interpretation that, at pH 7, pigment IV is characterized by a protonated Asp^{85}.

Alteration in the pK_a of Asp^{85} was also observed for pigment V in which the purple to blue transition is characterized by a pK_a of 4.0 ± 0.1 in 0.1 M NaCl. The protonated Schiff base pK_a was measured as 11.5 for IV and 12.1 for pigment V. A similar reduction in the pK_a of IV was previously reported for bacterioopsin expressed in *Escherichia coli* incubated with chromophore **4** in micelles (Bhattacharya et al., 1992).

The isomer composition of pigment VI derived from chromophore **6**, which bears a five-membered ring bridging carbons 11 and 13, is mainly 13-*cis* (Albeck et al., 1986). The purple to blue transition of this pigment has a pK_a transition of 3.4 ± 0.1 in 0.1 M NaCl and is titrated from 522 to 460 nm with a pK_a of 12.0 ± 0.2. The pK_as of both transitions are relatively close to those observed for WT bR.

To further check the effect of steric perturbation in the retinal skeleton on the pK_a of the protonated Schiff base and Asp^{85}, we prepared pigment VII derived from chromophore **7** and characterized by a short polyene chain and an aromatic core in a position equivalent to carbons 8−10 in the native retinal polyene. The pK_a of the protonated Schiff base in solution (MeOH/water, 1:1) is similar to that of the protonated retinal Schiff base (7.0 ± 0.1). Titration of the artificial pigment VII indicated several transitions as shown in Figures 5 and 6. The purple (580 nm) to blue (618 nm) transition is characterized by a pK_a of 5.2 ± 0.1 (Figure 5). Following

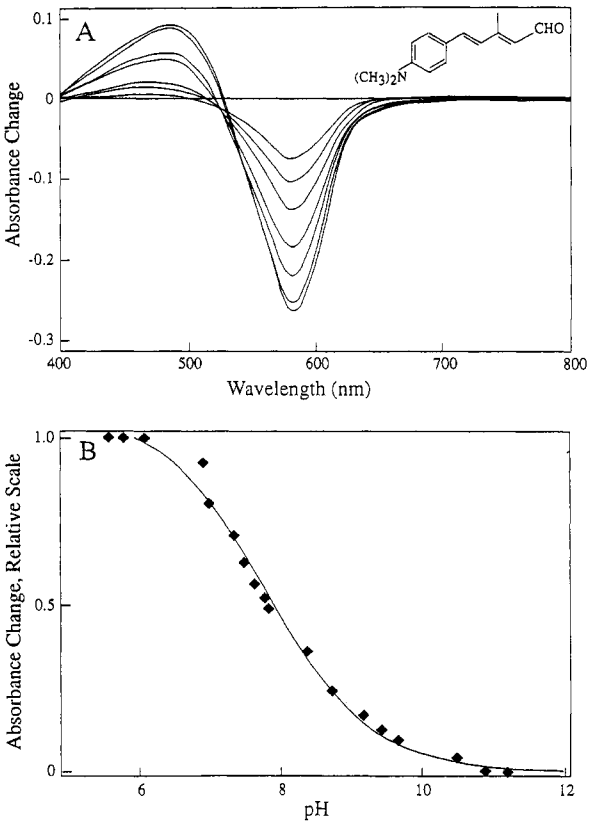


FIGURE 6: Titration of the high-pH transition in pigment VII. (A) Difference absorption spectra at $(pH)_i$ − pH 6.1. Spectra are shown for pH 7.35, 7.5, 7.8, 8.4, 8.75, 9.45, and 9.65. The titration was carried out in 0.5 M NaCl by adding 0.1 N NaOH solution. (B) pH dependence of absorption change monitored at 580 nm. The solid line represents the best-fit titration with a pK_a of 7.8 ± 0.1.

pH elevation, the 580-nm absorption was blue-shifted to 496 nm, exhibiting a pK_a of 7.8 ± 0.1 (Figure 6). Further pH elevation shifted the absorption maximum to 390 nm with a corresponding pK_a of 12.0 ± 0.2.

DISCUSSION

It has been suggested that the red shift associated with the purple to blue transition of bacteriorhodopsin is associated with the titration (protonation) of Asp^{85} (Metz et al., 1992), while deprotonation of the Schiff base (in the dark) leads to a significant blue shift in the absorption maxima (Druckmann et al., 1982). These characteristic changes in the absorption allow monitoring of the pK_a of these groups by following the respective absorption maxima.

The present study demonstrates that it is possible to affect the pK_a values of the protonated Schiff base and Asp^{85} by using sterically modified chromophores. The pK_a changes are much larger than those attributable to changes in the pK_a intrinsic values of the respective chromophores and are, therefore, due to changes in protein−chromophore interactions with respect to the native pigment. The most significant alterations in the pK_a of these artificial pigments relative to native bR were observed in pigments II and III, derived from 13-demethyl-11,14-epoxyretinal (**2**) and 13-demethyl-9,12-epoxyretinal (**3**). We suggest that the five-membered ring forces a significant distortion in the polyene skeleton, thereby altering the alignment of the Schiff base linkage and Asp^{85} relative to wild-type bR. This spatial difference is reflected in a major alteration of the pK_a of the protonated Schiff base, which is reduced by ca. 4 and 3 pK_a units relative to bR for pigments II and III, respectively. Taking into account the intrinsic pK_a change of the retinal analogs due to the five-membered ring, the net alteration in the pK_a due to the proposed geometrical change is ca. 2.7 units for both pigments.

The fact that both pigments II and III did not show a purple to blue transition as deduced from a lack of any change in the absorption maximum in the pH range of 1−9 suggests that Asp^{85} is protonated over this whole pH range and, thus, that its pK_a is ≥9. This suggestion is supported by the observation that the absorption detected for pigments II and III is very similar to that of the artificial pigments derived from the same two chromophores (**2** and **3**) and the mutant D85N. This behavior differs from that of the corresponding pigments carrying an *all-trans*-retinal. Thus, at pH 7, D85N exhibits a red shift in the spectrum relative to native bR, due to replacement of the negatively charged Asp^{85} by the neutral residue asparagine. Lack of a red shift in the artificial pigments bearing the five-membered ring supports the suggestion that Asp^{85} is protonated in pigments II and III, namely, the negative charge is absent even without replacing the Asp^{85} residue. In keeping with this conclusion, flash photolysis studies of pigment III at pH 7 (Sheves et al., 1985) revealed a photocycle reminiscent of that of the blue membrane and the D85N mutant (Thorgeirsson et al., 1991). Namely, the M intermediate, which is known to be due to proton transfer from the Schiff base to Asp^{85}, was not formed at pH 7. This is consistent with the inability of the protonated form of Asp^{85} to serve as a proton acceptor. Finally, we note that the absorption maxima of pigments II and III (568 and 570 nm, correspondingly), assumed to be associated with a protonated Asp^{85} counterion, are significantly blue shifted relative to the blue membrane of the wild type (605 nm). This behavior is probably due to a twist around single bonds of the polyene chain induced by the five-membered ring. This interpretation is supported by recent molecular dynamic simulations (Humphrey et al., 1994) indicating that the retinal chromophore tends to adopt single-bond rotations in the bR binding site upon polyene substitution with bulky groups.

We suggest that Asp^{85} is protonated at neutral pH also in pigment IV. This pigment shows a blue shift in absorption above pH 7 (pK_a ≅ 8), which replaces the blue to purple transition of native bR, and which we thus attribute to the titration of Asp^{85}. The incorporation of the five-membered ring spanning carbons 12−14 forces bond 12C−13C to adopt a planar conformation, which might alter the geometrical arrangement in the binding site. This interpretation is consistent with the finding that the retinal 14C experiences a close contact with its binding site environment, as indicated by the fact that incorporation of a methyl group at this position completely prevents pigment formation (Schiffmiller et al., 1985). Analogously, pigment IV is formed only following incubation of the retinal analog with the opsin for 10 days (Fang et al., 1983). This is in keeping with steric perturbations in the vicinity of 14C, which in turn might result in a different geometrical alignment of the protonated Schiff base linkage, Asp^{85}, and bound water molecules which bridge these residues. We note that at this stage we cannot completely exclude a global change in the protein conformation, which can affect the environment of Asp^{85}.

Pigment V exhibits a pK_a alteration of D85, which is considerably smaller than pigment IV, whereas in pigment VI a very small change relative to native bR is detected. Such alterations should be considered in terms of the retinal conformation in the binding site. It has been suggested on the basis of ^2H NMR that the retinal chromophore in the bacteriorhodopsin binding site has an in-plane curvature (Ulrich et al., 1994). This conformation was also suggested by molecular dynamic calculations, which indicate a twist around the 12C−13C single bond (Humphrey et al., 1994). The rings in pigments V and VI prevent rotation around this bond. However, the alteration in the pK_a of Asp^{85} and the protonated Schiff base linkage is smaller than that observed for pigment IV. This result indicates that the 14C position is much more sensitive to chromophore−protein interaction than positions 11 and 13 and, therefore, that the perturbations induced a ring spanning carbons 12−14 are more significant than those induced by a ring spanning carbons 11−13. The dramatic alterations induced in pigments II and III are probably mainly due to the significant distortion of the retinal skeleton, due to the furan ring, whereas in the other pigments the retinal skeleton experiences only minor alterations.

Alteration of the pK_a of Asp^{85} in the case of pigment VII, to $pK_a = 5.2 \pm 0.1$, is interpreted in terms of its aromatic core, which introduces steric perturbation in the vicinity of 7C−10C of the retinal skeleton. The second pH-induced spectral transition is characterized by a pK_a of 7.8 ± 0.1 and a significant blue shift in the absorption maximum (from 580 to 496 nm). Since the 496-nm absorption is quite red-shifted for a non-protonated Schiff base, it is probably not associated with deprotonation of the Schiff base. The significant blue shift might be caused by a distinct alteration of the binding site structure, resulting in a decrease in the Schiff base−counterion distance. It is worthwhile noting that a minor red shift of the purple membrane was observed above pH 8 and a partially strong blue shift to 490 nm was observed above pH 10 (Balashov et al., 1991). It is known that one or more protein residues with a pK_a of ~9 affects the photocycle of *trans*-bR (Ort & Parson, 1978; Rosenbach et al., 1982; Hanamoto et al., 1984; Liu, 1990), as well as its 13-*cis* photocycle (Drachev et al. 1993, Steinberg et al. 1993) and the proton-pumping activity (Kono et al., 1993). Future studies should identify these residues and clarify whether one or more of them are identical to the group or groups responsible for the significant blue shift observed in pigment VII.

An interesting phenomenon revealed in the present study is associated with the relative effects of the synthetic chromophores on the pK_a of the protonated Schiff base and Asp^{85}, respectively. The geometrical perturbation in the binding site affects both residues, which appear to influence each other. However, the effect on Asp^{85} is considerably more significant. We suggest that geometrical perturbation in the binding site affects the bound water structure, dramatically increasing the pK_a of Asp^{85}. The effect is assigned to the efficient hydrogen bonding of this residue with water molecules, which is a dominant factor in controlling its pK_a. The pK_a of the protonated Schiff base is influenced as well. However, the most significant alteration is observed in pigments II and III, which are the only ones in which the Schiff base titration is carried out when D85 is presumed to be protonated. Thus, alteration of the Schiff base pK_a in these pigments appears to be a consequence of the protonation of the Asp^{85} residue, which abolishes a major electrostatic stabilization of the protonated Schiff base. The importance of bound water in controlling the pK_a of residues in the binding site of bacteriorhodopsin and the interplay between the pK_a of the protonated Schiff base and Asp^{85} were recently supported by theoretical calculations (Sampogna & Honig, 1994).

The results of the present study strongly support the recently proposed model that attributes the high pK_a of the protonated retinal Schiff base and the low pK_a of Asp^{85} to a well-defined structure of the two residues that allows bound water to bridge the two groups and to stabilize the ion pair (Gat & Sheves, 1993). This model suggested that, following light absorption, this defined structure is perturbed, thereby inducing proton transfer from the Schiff base to Asp^{85} due to ion pair destabilization. A newly established hydrogen-bonding network might further stabilize the light-induced structure. Recent studies with bR mutants A53V and V49A have supported the possibility that the changed geometrical relationship of the protonated Schiff base and Asp^{85} after photoisomerization is a possible reason for the proton transfer (Brown et al., 1994). In addition, we note that molecular dynamic simulations of bacteriorhodopsin confirm the postulated bound water bridge between the protonated Schiff base hydrogen and Asp^{85} (Humphrey et al., 1994), as well as recent FTIR studies (Maeda et al., 1994). We note that, although the present studies support the donor−water−acceptor arrangement as an important factor controlling the pK_a and proton transfer, proof is not yet conclusive, and a global protein conformational alteration can be induced by the retinal analogs. Future studies should clarify this issue.

ACKNOWLEDGMENT

We thank Prof. J. Lanyi for reading the manuscript and for valuable comments. We are indebted to Prof. R. Needleman and Prof. J. Lanyi for the generous gift of D85N mutant.

REFERENCES

Albeck, A., Friedman, N., Sheves, M., & Ottolenghi, M. (1986) *J. Am. Chem. Soc. 108*, 4614−4619.

Baasov, T., & Sheves, M. (1986) *Biochemistry 25*, 5249−5257.

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

Bhattacharya, S., Marti, T., Otto, H., Heyn, M. P., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 6757−6762.

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

Brock, A. D., Muradin-Szweykowska, M., Courtin, J. M. L., & Lugtenburg, J. (1983) *Recl. Trav. Chim. Pays-Bas 102*, 46−51.

Brown, L., Bonet, L., Needleman, R., & Lanyi, J. K. (1993) *Biophys. J. 65*, 124−130.

Brown, L., Gat, Y., Sheves, M., Yamazaki, Y., Maeda, A., Needleman, R., & Lanyi, J. K. (1994) *Biochemistry 33*, 12001−12011.

Butt, H., Fendler, K., Der, A., & Bamberg, E. (1989) *Biophys. J. 56*, 851−859.

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

Drachev, L. A., Dracheva, S. V., & Kaulen, A. D. (1993) *FEBS Lett. 332*, 67−70.

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

DuPuis, P., Harosi, I., Sandorfy, C., Leclerq, J., & Vocelle, D. (1980) *Rev. Can. Biol. 39*, 247−258.

Ebrey, T. G. (1993) in *Thermodynamics of Membranes, Receptors and Channels* (Jackson, M., Ed.) pp 353−387, CRC Press, New York.
Fang, J., Carriker, J., Balogh-Nair, V., & Nakanishi, K. (1983) *J. Am. Chem. Soc. 105*, 5162−5164.
Friedman, N., Albeck, A., Sheves, M., & Ottolenghi, M. (1989) *Biophys. J. 56*, 1259−1265.
Gat, Y., & Sheves, M. (1993) *J. Am. Chem. Soc. 115*, 3772−3773.
Gerwert, K., Souvignier, G., & Hess, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9774−9778.
Hanamoto, J., DuPuis, P., & El-Sayed, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7083−7087.
Hildebrant, P., & Stockburger, M. (1984) *Biochemistry 23*, 5539−5548.
Honig, B., Ebrey, T. G., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503−2507.
Humphrey, W., Lugonov, I., Schulten, K., & Sheves, M. (1994) *Biochemistry 33*, 3668−3678.
Jonas, R., & Ebrey, T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 149−153.
Kono, M., Misra, S., & Ebrey, T. G. (1993) *FEBS Lett. 331*, 31−34.
Lanyi, J. K. (1993) *Biochim. Biophys. Acta 1183*, 241−261.
Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462−4466.
Liu, S., (1990) *Biophys. J. 57*, 943−950.
Longstaff, C., & Rando, R. (1987) *Biochemistry 26*, 6107−6113.
Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462−467.
Maeda, A., Sasaki, J., Yamazaki, Y., Needleman, R., & Lanyi, J. (1994) *Biochemistry 33*, 1713−1717.
Mathies, R. A., Lin, S. W., Ames, J. B., & Pollard, W. T. (1991) *Annu. Rev. Biophys. Bioeng. Chem. 20*, 491−518.
Metz, G., Siebert, F., & Engelhard, M. (1992) *FEBS Lett. 303*, 237−242.
Oesterhelt, D., Tittor, J., & Bamberg, E. (1992) *J. Bioenerg. Biomembr. 24*, 181−191.
Ort, D., & Parson, W. (1978) *J. Biol. Chem. 253*, 6158−6164.
Ottolenghi, M., & Sheves, M. (1989) *J. Membr. Biol. 112*, 192−212.
Rosenbach, V., Goldberg, R., Gilon, C., & Ottolenghi, M. (1992) *Photochem. Photobiol. 36*, 197−201.
Sampogna, R., & Honig, B. (1994) *Biophys. J. 66*, 1341−1352.
Scheiner, S., & Hillebrand, E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2741−2745.
Scheiner, S., & Duan, X. (1991) *Biophys. J. 60*, 874−883.
Schiffmiller, R., Callender, R. H., Waddell, R., Govindjee, R., Ebrey, T. G., Kakitani, H., Honig, B. H., & Nakanishi, K. (1985) *Photochem. Photobiol. 41*, 564−567.
Schulten, K., & Tavan, P. (1978) *Nature (London) 272*, 85−87.
Sheves, M., Friedman, N., Albeck, A., & Ottolenghi, M. (1985) *Biochemistry 24*, 1260−1265.
Sheves, M., Friedman, N., Albeck, A., & Ottolenghi, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3262−3266.
Steinberg, G., Sheves, M., Bressler, S., & Ottolenghi, M. (1994) *Biochemistry 33*, 12439−12450.
Stern, L. J., Ahl, P., Marti, T., Mogi, T., Dunach, M., Berkowitz, S., Rothschild, K. J., & Khorana, H. G. (1989) *Biochemistry 28*, 10035−10042.
Thorgeirsson, T., Milder, S., Miercke, L., Betlach, M., Shard, R., Stroud, R., & Kliger, D. S. (1991) *Biochemistry 30*, 9133−9142.
Tokunaga, F., & Ebrey, T. (1978) *Biochemistry 17*, 1915−1922.
Ulrich, A., Watts, A., Wallat, I., & Heyn, M. P. (1994) *Biochemistry 33*, 5370−5375.
Warshel, A., Russel, S., & Churg, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4785−4789.

BI950213E