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A Covalent Link between the Chromophore and the Protein Backbone of Bacteriorhodopsin Is Not Required for Forming a Photochemically Active Pigment Analogous to the Wild Type†

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ABSTRACT: Bacteriorhodopsin pigments lacking the retinal–Lys-216 covalent bond were prepared by reconstituting the K216G mutant protein with retinal alkylamine Schiff bases. The procedure follows the approach of Zhukovsky *et al.* [Zhukovsky, E., Robinson, P., & Oprian, D. (1991) *Science 251*, 558–560] in the case of visual (rhodopsin) pigments. Reconstitution leads to a mixture of three pigments. One of them, bR(K216G)/566a, absorbs (pH = 6.9) at 566 nm. Its absorption is pH-dependent, exhibiting a purple to blue transition. The pigment's laser-induced photocycle patterns are similar to those of wild-type *all-trans*-bR. A second component, bR(K216G)/566b, exhibits an independent photocycle reminiscent of that of wild-type 13-*cis*-bR. A third pigment component, bR(K216G)/630, absorbs around 630 nm. Experiments in the presence of a pH dye indicator show that illumination of bR(K216G)/566 produces a detectable proton gradient. It is concluded that a covalent bond between the retinal chromophore and the protein backbone is not a prerequisite for the basic structure and photochemical features of bR or for its proton pump activity.

Visual pigments such as rhodopsin and photosynthetic bacteriorhodopsin (bR)[1] share a similar retinal chromophore which is covalently bound via a protonated Schiff base linkage to the ε-amino group of a lysine protein residue. The chromophore–protein systems are 11-*cis*-retinal bound to Lys-296 in the case of rhodospin and *all-trans*- (or 13-*cis*-) retinal bound to Lys-216 in the case of bacteriorhodopsin [for reviews, see Ottolenghi and Sheves (1989), Birge (1990), Mathies *et al.* (1991), and Ebrey (1993)].

The question as to whether the covalent retinal–protein bond in rhodopsins plays a role in controlling the photocycles and the biological function of the pigments is intriguing. In this regard it is interesting to note that G-protein receptors, other than visual pigments, bind their agonists through noncovalent interactions. In a pioneering work Zhukovsky *et al.* (1991) have prepared rhodopsin pigments by incorporating

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; K216G, bacteriorhodopsin in which lysine 216 is substituted with glycine.

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11-*cis*-retinal Schiff bases into the binding pockets of two rhodopsin mutants, K296G and K296A, in which a covalent retinal–opsin bond in the binding pocket cannot be formed. The absorption spectra of both pigments were found to be close to those of the wild type. Moreover, following light absorption, the *n*-propylamine Schiff base–K296G pigment was found to activate the G-protein transducin with a 30–40% efficiency with respect to the wild-type protein. It was concluded that the retinal–lysine covalent bond is not essential for binding the chromophore or for triggering the visual response.

In the case of bacteriorhodopsin the primary photon-induced events are associated with isomerization of the 13–14 retinal double bond. This isomerization would lead to significant problems in the accommodation of the new chromophore structure in the retinal pocket. An early suggestion to resolve this problem was introduced by Schulten and Tavan (1978), who proposed that the C_{14}–C_{15} single bond could simultaneously rotate during the photochemical event in order to relieve the chromophore strain. However, this suggestion has been challenged by resonance Raman data (Smith *et al.*, 1986) that did not detect rotation around the C_{14}–C_{15} bond. An important degree of freedom for accommodation of a chromophore change can be provided by the lysine residue which is covalently linked to the retinal chromophore. In the past decade there have been numerous studies on light-induced structural changes in the retinal chromophore. However, only recently has experimental information on the conformational changes in the lysine residue and on its role in the photochemical events become available. Thus, using Fourier transform infrared (FTIR) spectroscopy it was shown that the lysine chain experiences conformational changes following light absorption by the retinal chromophore (McMaster & Lewis, 1988). Moreover, it appears that modes of the retinal protonated Schiff base are coupled to the carbons of the lysine chain and also to the α-carbon on the lysine backbone (Gat *et al.*, 1992).

With the purpose of investigating the role of the retinal–Lys-216 bond in establishing the molecular structure of the binding pocket and in determining the photobiological activity of bR, we prepared a noncovalent pigment derived from the K216G mutant. We show that a pigment is formed upon reconstitution with *all-trans*-retinal Schiff bases. We also present data concerning the spectroscopy of bR (K216G) and, for the first time, describe the photocycle of a noncovalent rhodopsin pigment. We note that the successful preparation of a noncovalent bR pigment derived from the K216A mutant has also been recently reported by Schweiger *et al.* (1993).

MATERIALS AND METHODS

The K216G Mutant. The *Halobacterium halobium* strain carrying a gene with a Lys-216 → Gly substitution in bacteriorhodopsin was constructed by transformation with halobacterial plasmid. The replicating shuttle vector used, pXL–Nov^r, contained the Tet^r for selection in *Escherichia coli* and gene Nov^r which confers resistance to novobiocin in *H. halobium*. PXL–Nov^r will be described in more detail elsewhere.

The cells were grown in the usual medium (Oesterhelt & Stoeckenius, 1974), first (100 cm^3) in the presence of novobiocin (1 μg/1 cm^3) and then diluted to 2 L of medium and grown without novobiocin. The membranes were prepared from the cultures as described before (Oesterhelt & Stoeckenius, 1974).

Retinal Schiff Base Preparation and Pigment Titrations. Condensation of *all-trans*-retinal with 2 equiv of the appropriate amine was carried out in ethanol at 25 °C for 30 min. Evaporation under reduced pressure removed excess amine and solvent, yielding the desired retinal Schiff base. Pigment titrations were carried out at 0 °C (to avoid significant decomposition) using appropriate buffers.

Pulsed Laser Photolysis. Pulsed laser photolysis was carried out as previously described (Druckmann *et al.*, 1993) using a PRA N_2-dye laser system, monitoring with a pulsed or continuous 75-W Xe source. Signals were recorded using a photomultiplier and a 2440 Tektronix digital oscilloscope. Interfering effects due to the monitoring beam were minimized by placing an interference filter between the lamp and the reaction cell 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. In addition, the sample was replaced after each set of pulses. The pH indicator pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt; purchased from Eastman Kodak) was used in the suspensions exposed to pulsed laser experiments without further purification. All experiments were carried out at pH = 6.9.

RESULTS AND DISCUSSION

Reconstitution of bR(K216G) with Retinal Schiff Bases. Reconstitution of bR(K216G) was carried out by adding an ethanol solution (1% volume) of the retinal Schiff base to a suspension of membranes from the mutant at 25 °C. The retinal Schiff bases were derived from methyl-, ethyl-, *n*-propyl-, or *n*-butylamines. Figure 1a shows the effect of the addition of the retinylidene ethylamine Schiff base to a K216G preparation. Addition of the Schiff base results in a gradual change in absorbance represented by the difference spectra recorded at various times as shown in Figure 1b. It is evident that two new bands with different evolution rates (as can be deduced from Figure 1b,c) are observed, peaking at 566 and 630 nm. The same phenomena are also observed with the methyl-, propyl-, and butylamine Schiff bases. It is thus evident that two pigments are formed with a purple (566-nm) to blue (630-nm) ratio of 7:4, which are not equilibrated over time scales of at least several hours. The pigments experience thermal decomposition to a species absorbing around 460 nm. The process, occurring within a few hours at 25 °C, is enhanced by exposure to light.

In variance with native bR, the absorption spectrum of the bR(K216G) mutant exhibits an absorption at ca. 460 nm (Figure 1a), which shifts to 390 nm upon addition of hydroxylamine in the dark. Formation of the 566- and 630-nm pigments upon addition of retinal Schiff bases is observed independently of whether or not the K216G samples are pretreated with hydroxylamine. The reaction with hydroxylamine may be accounted for by attributing the 460-nm band to the presence of retinal in the binding pocket. Hydroxylamine reacts with retinal, yielding an oxime which absorbs around 360 nm. This interpretation is in keeping with independent studies invoking a substantial red shift between bacterioopsin-bound retinal and retinal in solution (Schreckenbach *et al.*, 1978). Alternatively, the 460-nm band may be due to a retinal Schiff base with ϵ-amino groups of a lysine other than Lys-216 or to a retinaldehyde molecule bound to the protein noncovalently in a region other than the usual binding site.

The absorption spectrum of wild-type bR is known to be markedly affected by pH. A major feature (Fischer &

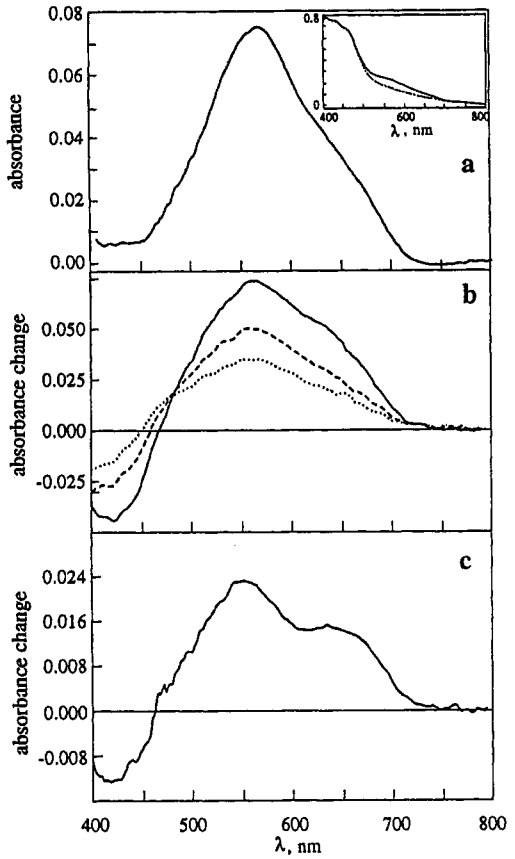

FIGURE 1: (a) Absorption spectrum of the bR(K216G)–ethylamine Schiff base pigment obtained by subtracting the spectrum before and after addition of retinal Schiff base. Insert: the spectrum of bR(K216G) before addition of 1 equiv of retinal ethylamine Schiff base (- - -) and after (—). (b) Difference spectra after the addition of the Schiff base as a function of time. A spectrum that was taken 2 s after the addition of the retinal Schiff base was subtracted from spectra at different delay times. The delay times are 10 s (curve ···), 20 s (curve - - -), and 90 s (curve —). (c) Difference spectrum between the 90- and 20-s curves.

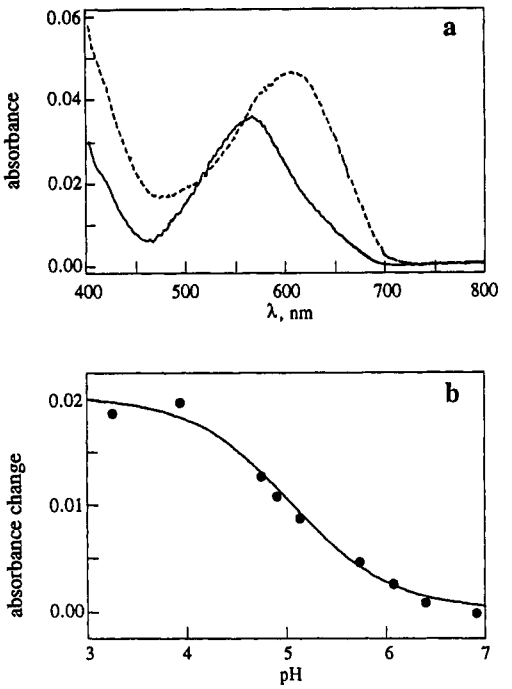

FIGURE 2: pH effects on the absorption spectrum of the bR(K216G)–ethylamine Schiff base. (a) Absorption at pH = 6.9 (—) and pH = 3.5 (- - -). (b) pH dependence of absorption changes at 620 nm in the bR(K216G)–ethylamine Schiff base at 0 °C. The solid line represents the best fit titration curve with pK_a = 5.0 and n = 0.89.

Oesterhelt, 1979; Mowery *et al.*, 1979; Kimura *et al.*, 1985; Jonas & Ebrey, 1991) is the purple (568-nm) to blue (605-nm) transition induced at low pH, exhibiting an apparent pK_a of 3.1 ± 0.1 in water. The purple ↔ blue transition is interpreted in terms of titration of the Asp-85 residue, which interacts with the polyene via electrostatic and H-bonding interactions. Figure 2 shows that a similar pH effect is also observed in the case of the 566-nm ethylamine Schiff base pigments of bR(K216G). Upon reducing the pH the 566-nm band is replaced by one at 620 nm. The transition exhibits a pK_a value of 5.0 ± 0.1 in water (Figure 2b), which is ca. 2 units higher than that of the wild type.

The above observations clearly indicate that upon reconstitution with alkylamine Schiff bases K216G yields a pigment, denoted in the case of ethylamine as bR(K216G)–EASB, which closely resembles the wild type both in its spectrum and in its blue ↔ purple interconversion. Attributing the latter to titration of the same amino acid residue as in the wild type leads to the conclusion that removal of the retinal–protein backbone covalent bond has perturbed the binding pocket, in a way that increased the pK_a of Asp-85 by approximately 2 pK_a units. It has recently been suggested (Gat & Sheves, 1993) that the pK_a of the protonated Schiff base linkage and that of Asp-85 are controlled by a specific angle between the two residues, which allows water molecules to bridge the two groups and to stabilize the ion pair. Other protein residues can participate through hydrogen bonding in the ion pair stabilization. Perturbation of the binding pocket by eliminating the covalent bond of the Schiff base linkage to the protein may alter the hydrogen-bonding network, modifying the pK_a of binding site residues. This mechanism can account for the Asp-85 pK_a change observed in the 566-nm pigment. In this respect we note that the pK_a of residue 85 is also raised in the D85E mutant (Lanyi *et al.*, 1992), as well as when R82 is replaced by alanine. Both observations are indicative of the importance of electrostatic interactions in determining the pK_a values in the binding pocket (Brown *et al.*, 1993; Balashov *et al.*, 1993).

It is difficult at present to account for the 630-nm pigment, bR(K216G)/630, which does not have an analogous counterpart in wild-type bR. A feasible possibility is that, in the same binding pocket, the retinal polyene assumes two different conformations or configurations characterized by different spectra. This would be reminiscent of the behavior of artificial bR pigments derived from synthetic retinals carrying bulky substituents at the ring C_4 position (Sheves *et al.*, 1984). Analogously to bR(K216G)–EASB, these proteins exhibit two nonequilibrated pigments characterized by different spectra and by independent photocycles. Another alternative would be to assume the occurrence of two protein conformers, differing in the structure of their binding pocket and thus in the retinal–protein interactions, which determine the spectrum of the pigment.

Photocycle and Proton Translocation Activity of K216G. Suspensions of bR(K216G)–EASB (pH 6.9) were submitted to pulsed laser photolysis using 536-nm excitation. The first photointermediate observed with our (~10-ns) time resolution is a red-shifted species decaying with a half-life of ~280 ns (Figure 3a). The transient absorption spectrum recorded after termination of this primary decay is shown in Figure 4a. It consists of an increased absorbance in the red coupled with bleaching around 550 nm, which corresponds to the absorption of K216G/566. The subsequent transient events consist of a continuous increase (280-μs half-life time) of a blue-shifted

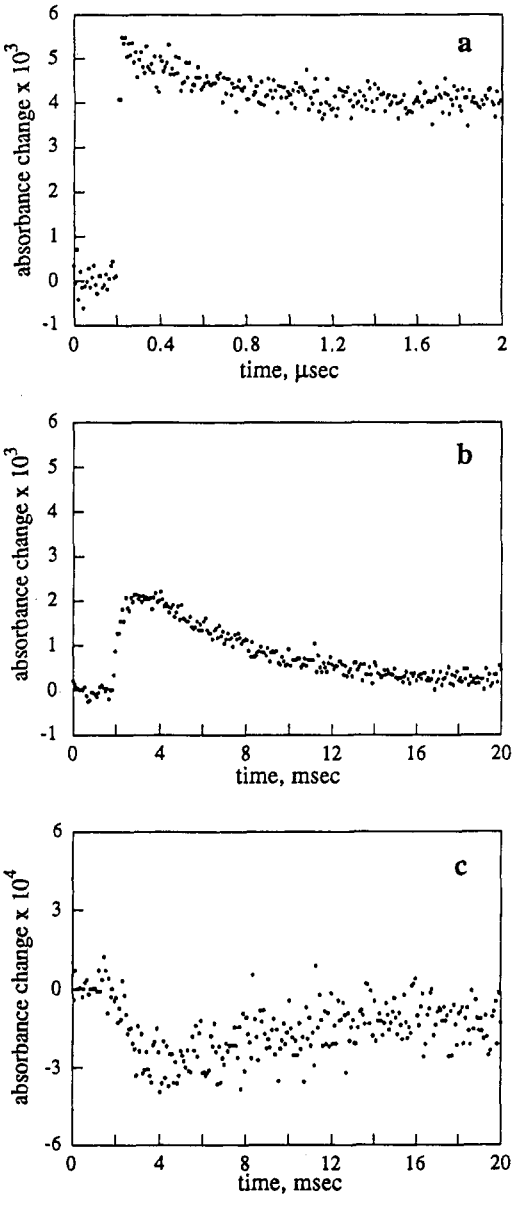

FIGURE 3: Characteristic (536-nm) laser-induced absorbance profiles of bR(K216G)–EASB. (a) Decay of a red-absorbing (K) intermediate at 630 nm. (b) Rise and decay of the blue-shifted M intermediate at 410 nm. (c) Proton release/uptake measured at 456 nm with a pyranine dye at pH = 7.2, unbuffered, and 25 °C. The trace represents the difference between the absorbance change profile in the above solution and that induced in an analogous solution lacking the pyranine indicator.

species peaking at 410 nm (Figure 3b). Subtraction of the 40-μs spectrum from the 1.7-ms spectrum in Figure 4a yields the difference spectrum between the blue-shifted intermediate and its photocycle precursor (Figure 4b). Except for the small contribution of the shoulder around 630 nm this difference spectrum very closely resembles that between M and L in the photocycle of the wild type. These events are followed by a slow ($\tau_{1/2}$ = 4.4 ms) decay, which corresponds to the difference spectrum shown in Figure 5 (the latter is obtained by a steady-state illumination experiment). This difference spectrum represents a 566-nm purple → red transformation which is reversed to the original absorption of the K216G pigment over a time scale of 2 s.

The above observations clearly indicate that bR(K216G) exhibits a photocycle with patterns which are closely reminiscent of that of the light-adapted (all-trans) wild type. However, in variance with the wild type, a single unbranched

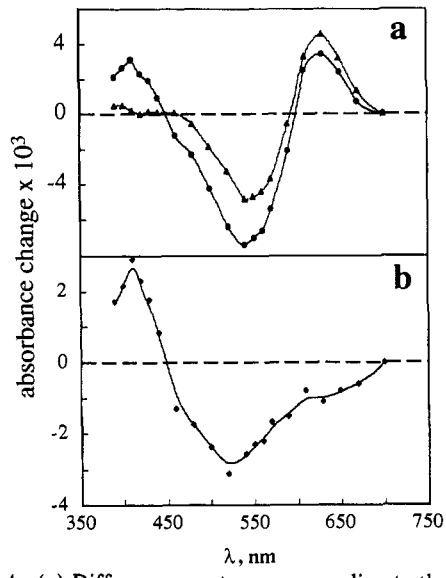

FIGURE 4: (a) Difference spectra corresponding to the experiment described in Figure 3, recorded 40 μs (▲) and 1.7 ms (●) after the pulse, respectively. The trace in (b) represents the difference between (●) and (▲).

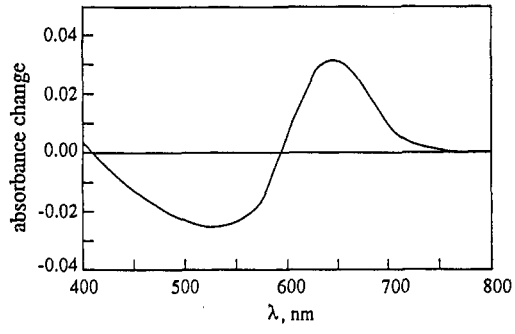

FIGURE 5: Light-induced changes in bR(K216G)–EASB recorded using steady-state excitation (500-nm interference filter). The difference spectrum represents the final slow stage (2 s) of the photocycle events described in Figures 3 and 4.

photocycle, e.g.

$$\text{bR(K216G)} \xrightarrow{h\nu} \text{K} \rightarrow \text{L} \rightarrow \text{M} \rightarrow \text{bR(K216G)}$$

with or without reversible (equilibrium) steps is not feasible. Thus, as can be seen in Figure 4a a substantial red-shifted absorption is still present at the 40-μs "L" stage, which does not transform into the blue-shifted M species. Our data are, however, in keeping with a superposition of two independent (uncoupled) photocycles of two bR(K216G)/566 pigments (a and b), both absorbing around 566 nm, according to the schemes

$$\text{bR(K216G)/566a} \rightarrow \text{K} \rightarrow \text{L} \rightarrow \text{M} \rightarrow \text{bR(K216G)/566a} \quad (1)$$

$$\text{bR(K216G)/566b} \rightarrow \text{P}_{630} \rightarrow \text{bR(K216G)/566b} \quad (2)$$

where K, L, and M represent the three observed stages in photocycle 1 and P_{630} represents the single (slow) stage of photocycle 2. Accordingly, photocycle 1 will be responsible for the $\tau_{1/2}$ = 280-ns decay in the red (K → L transition), for the subsequent continuous increase at 410 nm and depletion at 550 nm (L → M), and for the $\tau_{1/2}$ = 4.4-ms decay at 410 nm (which completes the photocycle). We note that the 630-nm shoulder, which contributes to the M – L difference spectrum in Figure 4b, is absent in the wild-type photocycle.

It may be attributed to a residual contribution of the K intermediate (equilibrated with L) or alternatively to the contribution of an independent photocycle of bR(K216G)/630. Assuming that the rise of P_{630} is faster than our 10-ns time resolution, photocycle 2 will account for the presence of a red-shifted intermediate P_{630} over the whole time range, up to 2 s.

At present it is difficult to make unambiguous assignments of the 566- and 630-nm pigments. However, on the basis of the analogies with the wild type, it is tempting to identify bR(K216G)/566a and bR(K216G)/566b as the all-trans and 13-cis protonated Schiff base chromophores, respectively. Thus, the bR(K216G) photocycle closely resembles that of *all-trans*-bR, and in analogy to that of 13-*cis*-bR (Kalisky *et al.*, 1977), photocycle 2 exhibits a red-shifted long-lived intermediate, P_{630}, that decays to the original pigment without generating an M species. Obviously these assumptions must be confirmed by future studies establishing the isomer composition of (K216G)/566. As for the relative efficiency of M formation (i.e., of photocycle 1) the observed yield, normalized to the initial pigment absorbances at 530 nm, is 7 ± 2 times smaller than in light-adapted (wild-type) bR. Since we cannot at present estimate the relative amounts of bR(216G)/566a and bR(K216G)/566b, we are unable to accurately evaluate the efficiency of photocycle 1 relative to that of wild-type bR. However, considering the substantial contribution of photocycle 2, the efficiency of photocycle 1 and that of wild-type *all-trans*-bR appear to be compatible.

On the basis of the close resemblance of the photocycles of bR(K216G)/566a and the wild type, we carried out experiments in the presence of the pyranine pH indicator, aiming to establish the photoinduced proton-translocation capability of the noncovalent bonded pigment. Pulsed laser excitation of wild-type bR (pH 7.2, unbuffered) in the presence of pyranine dye yields characteristic transient (bleaching) absorbance changes at ~456 nm due to the sequential proton release (during the L → M transition) and proton uptake (during the N → O transition) processes (Grzesick & Dencher 1986; Herberle & Dencher 1990; Otto *et al.*, 1989; 1990; Zimanyi *et al.*, 1992). Analogous experiments were carried out for the K216G pigment around the 456-nm isosbestic point of Figure 4. Spectra in the absence of pyranine were subtracted from those in the presence of the pH indicator, yielding net effects as shown in Figure 3c. The figure clearly shows the presence of a light-induced dye signal which decays at a rate similar to that of the M intermediate of bR(K21G)/566a. We note that the signal is not observed when phosphate buffer (pH = 7.0) is added to the pigment suspension. Scaled to the amounts of the M intermediate generated in each case, we estimated the size of the transient change in the dye absorbance in the case of K216G to be of the same order of magnitude as that of the wild type. However, in view of our present signal/noise limitations imposed by the low effective yield of photocycle 1, a difference of 20–30% between the two proton pumping efficiencies cannot be completely excluded at this stage.

CONCLUSIONS

It is clearly evident that upon reconstitution with retinal Schiff bases K216G forms a pigment which lacks the covalent bond between the retinal Schiff base and the protein backbone. One component of this pigment [bR(K216G)/566a] is spectroscopically and photochemically similar to wild-type *all-trans*-bR and behaves analogously with respect to the blue ↔ purple transition attributed in both cases to the titration of Asp-85. The photocycle of this noncovalently bound pigment (photocycle 1) exhibits some differences with respect to the photocycle rates (half-life time) of the all-trans wild type: 280 ns vs 1.5 μs in the case of K → L, 280 μs vs 42 μs for L → M, and 4.4 ms vs 2.5 ms for the M → bR decay. Such observations imply that the retinal–protein interactions in the binding pocket are not markedly affected by the presence of the covalent attachment of the lysine chain to the protein backbone. This applies not only to the absorption spectrum but also to the photocycle patterns which are based on trans–cis isomerization about C_{13}–C_{14} (Braiman & Mathies, 1983; Fang *et al.*, 1983; Chang *et al.*, 1985). We note that molecular dynamic simulations suggested that the covalent link of the retinal to the protein does not affect the isomerization process significantly (Zhou et al., 1993). Apparently, the Schiff base moiety is firmly bound in the protein binding pocket, presumably via electrostatic and H-bonding interactions without requiring the covalent bond. However, the covalent bond appears to contribute to the pigment thermal and photochemical stability. Thus, the bR(K216G)/566 pigment decomposes at 25 °C either thermally within a few hours or following exposure to light. Moreover, if indeed it can be confirmed that the light-adapted form of bR(K216G)/566 contains a substantial amount of 13-cis isomer, it would imply that the retinal–protein covalent bond prevents a leak from the all-trans to the 13-cis photocycle. Nevertheless, it appears that the covalent bond does not impose serious restrictions on the retinal conformational changes and on the major retinal–protein interactions which characterize the (spectroscopy and the basic) photocycle pattern of bR. Moreover, the covalent bond is not a prerequisite condition for obtaining proton release and uptake following light absorption.

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