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Light-Induced Hydrolysis and Rebinding of Nonisomerizable Bacteriorhodopsin Pigment

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ABSTRACT Bacteriorhodopsin (bR) is characterized by a retinal-protein protonated Schiff base covalent bond, which is stable for light absorption. We have revealed a light-induced protonated Schiff base hydrolysis reaction in a 13-*cis* locked bR pigment (bR5.13; λ_{max} = 550 nm) in which isomerization around the critical $C_{13}{=}C_{14}$ double bond is prevented by a rigid ring structure. The photohydrolysis reaction takes place without isomerization around any of the double bonds along the polyene chain and is indicative of protein conformational alterations probably due to light-induced polarization of the retinal chromophore. Two photointermediates are formed during the hydrolysis reaction, H450 (λ_{max} = 450 nm) and H430 (λ_{max} = 430 nm), which are characterized by a 13-*cis* configuration as analyzed by high-performance liquid chromatography. Upon blue light irradiation after the hydrolysis reaction, these intermediates rebind to the apomembrane to reform bR5.13. Irradiation of the H450 intermediate forms the original pigment, whereas irradiation of H430 at neutral pH results in a red shifted species (P580), which thermally decays back to bR5.13. Electron paramagnetic resonance (EPR) spectroscopy indicates that the cytoplasmic side of bR5.13 resembles the conformation of the N photointermediate of native bR. Furthermore, using osmotically active solutes, we have observed that the hydrolysis rate is dependent on water activity on the cytoplasmic side. Finally, we suggest that the hydrolysis reaction proceeds via the reversed pathway of the binding process and allows trapping a new intermediate, which is not accumulated in the binding process.

INTRODUCTION

Light absorption by a retinal chromophore embedded in the bacteriorhodopsin (bR) binding site triggers a characteristic photocycle, which reflects mutual structural alterations in the retinal and its surrounding protein (for a series of reviews, see Ottolenghi and Sheves, 1995; Oesterhelt, 1998; Lanyi, 1999). Light-induced conformational changes in bR are the major driving force for the protein function as a light-driven proton pump (Subramaniam et al., 1999). These protein conformational changes, induced by retinal chromophore light absorption, were exclusively attributed to specific retinal $C_{13}{=}C_{14}$ double bond isomerization. An early analysis raised the possibility that the protein may respond to light-induced charge redistribution in the retinal chromophore (Lewis, 1978). In recent years, several studies have indicated that bR protein experiences light-induced conformational alterations even when isomerization around the critical $C_{13}{=}C_{14}$ double bond is prevented by a rigid ring structure. The first indication came from a study based on atomic force sensing technique, which monitors expansion in bR membranes, due to a laser pulse absorbed by the pigment. Similar signals were detected in artificial pigments that do not experience $C_{13}{=}C_{14}$ double bond isomerization or a photocycle (Rousso et al., 1997a). Later, chemical activity of bR and artificial pigments was used as a sensitive probe to monitor light-induced conformational changes at different domains of the protein. Light induced retinal-protein bond cleavage using hydroxylamine, monitored in bR and in "locked" artificial pigments, revealed light catalysis even when the $C_{13}{=}C_{14}$ bond was "locked" for isomerization (Rousso et al., 1998). Similarly, reduction and reoxidation reactions of a spin label probe were used to monitor light-induced conformational changes at the cytoplasmic side of the protein, in the vicinity of the 103 residue (Aharoni et al., 2000). This approach indicated as well that protein conformational alterations, which are not associated with the characteristic photocycle or with C═C isomerization are induced by light absorption. Moreover, the possibility that after light absorption by the "locked" pigments $C_{13}{=}C_{14}$ isomerization is replaced by another double bond was recently excluded by incorporating several dye molecules characterized by a completely rigid structure into bacteriorhodopsin. It was revealed that light-induced conformational alterations occur in the vicinity of the C103 residue even in completely nonisomerizable chromophores (Aharoni et al., 2001a). All such observations are consistent with protein structural alterations triggered by the primary light-induced polarization of the retinal or other polarizable moieties that reside in its binding site.

Binding of the retinal chromophore to bacterioopsin involves formation of two intermediates attributed to the retinal-protein complexes ("prepigments") absorbing at 400 nm and 430/460-nm complexes (Schreckenbach et al., 1977; Schweiger et al., 1994). It was suggested that in the first intermediate, the retinal is incorporated into the binding site without formation of the retinal-protein covalent bond,

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adopting a ring-chain planar conformation. The second intermediate absorbing at 430/460 nm, is characterized as well by a noncovalently bound retinal. The formation of the covalent protonated Schiff base bond is the rate-limiting step in the binding process controlled by a protein residue with a pK_a of 4.6 (Fischer and Oesterhelt, 1980). Moreover, it was demonstrated that the formation of the Schiff base linkage is inhibited at low humidity (Rousso et al., 1995, 1997b).

In the present study we reveal a new type of reaction, Schiff base hydrolysis, that is indicative of protein structural alterations, due to primary light-induced polarization of the retinal. The reaction efficiency is an exclusive property of the artificial pigment (bR5.13), derived from a 13-*cis* "locked" retinal in which $C_{13}=C_{14}$ double bond isomerization is prevented. Unlike native bR in which hydrolysis reaction is very unefficient, bR5.13 experiences an efficient light-induced hydrolysis of the protein chromophore Schiff base covalent bond. The reaction resembles the light-induced hydroxylamine reaction of bR but exhibits two interesting features. First, it is reminiscent of the hydrolysis reaction that characterized the photocycle of visual pigments. Second, it allows the detection of intermediates, which are not observed in the reversed chromophore-protein binding.

MATERIALS AND METHODS

Apomembrane preparation and reconstitution with 13-*cis* locked retinal

The apomembrane was prepared from bR, D85N, Y185F, and A103C mutants by reaction with hydroxylamine according to a previously described method (Oesterhelt et al., 1974). The various apomembranes were washed four times to eliminate hydroxylamine and incubated with 1.2 equivalents of the 13-*cis* locked retinal at pH 7, 25°C for 16 h.

Hydrolysis and rebinding reaction of 13-*cis* locked pigment (bR5.13)

Samples containing 1.5×10^{-6} M of 13-*cis* locked pigment and 7.5 mM of phosphate or borax buffer at various pH values were illuminated with orange light ($\lambda_{max} > 570$ nm) or blue light (420 nm $> \lambda_{max} >$ 470 nm). Hydrolysis reaction of the pigment with osmotically active solutes were carried out with 7.5 mM of borax buffer pH 9 containing 60% sucrose, 35% glucose, 80% glycerol, and 5% poly(diallyldimethylammonium chloride) (POAC) 20% (w/w). A partial, delipidated bR5.13 sample was prepared by 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) treatment according to a previously described method (Szundi and Stoeckenius, 1987). The hydrolysis reaction was performed with 7.5 mM borax buffer at pH 9. All reactions were carried out under identical illumination conditions using a halogen lamp with an output of 150 W equipped with a heat-absorbing filter. The illumination was kept steady for the whole experiment. Reaction rates of D85N 13-*cis* locked pigment could not be time-resolved by our experimental setup. Kinetic traces were followed at 550 nm (at the maximum of the pigment absorption) fitted to one and two exponential decay components according to the following equations: $y = a \cdot \exp(-kx)$ and $y = a_1 \cdot \exp(-k_f x) + (1 - a_1) \cdot \exp(-k_s x)$, respectively. a is the maximal relative value of the absorption difference at 550 nm between the pigment and the hydrolyzed products; k is the first order rate constant; a_1 is the fraction of the fast kinetic process, whereas k_f and k_s are the fast and the slow reaction rate constants, respectively. The fraction of the k_f component at different pH values was plotted and fitted to the equation: $y = a/(1 + 10^{n(pKa - pH)})$ in which a is the maximal relative value of the k_f, n is the number of protons participating in the transition, and pKa the midpoint of the observed transition.

High-performance liquid chromatography analysis

Extractions of the 13-*cis* locked chromophores were performed by mixing 200 μL of bR5.13 pigment ($\sim 8 \times 10^{-5}$ M) for 1 min with 500 μL EtOH precooled to 0°C. Five-hundred microliters of cold hexane was added, and the mixture was stirred for 2 min. The organic phase was separated, dried over magnesium sulfate, and evaporated to a volume of 20 μL. The high-performance liquid chromatography (HPLC) column was equilibrated with hexane/ethyl acetate (90:10 v/v or 65:35, v/v) for 1 h before sample injection. The extracted chromophores (in hexane) were analyzed by HPLC and eluted with hexane/ethyl acetate (90:10 v/v to analyze the aldehyde mixture, or 65:35 v/v to analyze the sodium borohydride-reduced mixture). The analysis was performed using an analytical column (LiChroCART, 250 mm $\times$ 8 mm, 5 μm; Merck, Rahway, NJ) at flow rate of 0.5 mL/min.

EPR measurements of spin-labeled bR and bR5.13

The spin label (1-oxyl-2, 2, 5, 5-tetramethylpyrroline-3-methyl) methanethiosulfonate was covalently attached to the cysteine residue of the A103C bR mutant and of its artificial pigment derived from chromophore 1 to yield the spin labeled side chain. A solution of 10 μL of 100 mM spin label (1-oxyl-2, 2, 5, 5-tetramethylpyrroline-3-methyl) methanethiosulfonate was added to 2 mL of suspension of 50 μM bR mutant and the artificial pigment in 0.1 M phosphate buffer (pH 8) and 0.1 M NaCl. The suspensions were stirred at room temperature for 14 h. The noncovalently bound spin label was removed by washing the membrane pellet four times with a solution of 1% bovine serum albumin. EPR spectra of the spin-labeled bR and bR5.13 were measured in the dark and under constant illumination with a 550-nm cutoff filter.

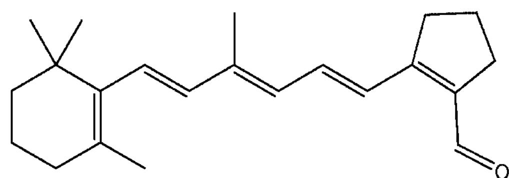
Absorption and EPR measurements

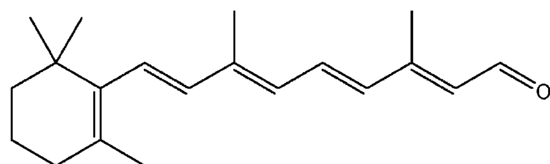
All ultraviolet/visible absorption measurements were carried out using a HP8453 spectrophotometer with a thermostated cell at constant temperature of 25°C. EPR measurements were performed on a Bruker ER200 DSRC spectrometer.

RESULTS

Light-induced hydrolysis of a locked 13-*cis* pigment

Pigment I, bR5.13, is derived from a retinal analog (**1**, Scheme 1) in which the 13-*cis*→ *trans* photoisomerization is prevented by a rigid ring structure. After irradiation ($\lambda >$ 570 nm), it experiences a significant blue shift in its long absorption band from 550 to 450 nm. (Fig. 1 *A*). The newly formed H450 intermediate was found to thermally decay to the H430-nm species, which can be detected either after a long period of illumination (800 s at neutral pH), or alternatively, after a short illumination and monitoring the sub-





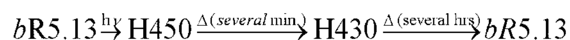
Scheme 1.

sequent thermal reaction (see Fig. 1 *B*). H430 reconverts thermally in ~5 h to the original pigment (bR5.13). The overall process is described in Scheme 2.

Blue light irradiation (420 nm $< \lambda <$ 470 nm) of both H450 and H430 species led to the generation of red-shifted bands that are indicative of a rebinding process. In the case of H450, the photoreaction leads to efficient reformation of the original absorption (Fig. 2). In contrast, illumination of the H430 resulted in a rebinding process that first produces a red shifted pigment P580 (λ_{max} = 580 nm), which subsequently undergoes a thermal transition to the original pigment, bR5.13 (550 nm, Fig. 3 *A*). At pH 9, the red shifted pigment is not detected (Fig. 3 *B*), whereas at pH 8 a mixture of the two pigments is observed (data not shown). We note that the reconstituted bR5.13 pigment also contains a retinal oxime originating from the bR bleaching process. However, because the retinal oxime absorbs at ~360 nm, it is not irradiated by the light wavelength used in the above experiments.

HPLC analysis of the reaction products

To characterize the illumination reaction products, we have carried out an HPLC analysis of the chromophores extracted from the bR5.13 and its various photochemically induced intermediates. The chromophore was extracted from a pigment that was incubated in the dark for 12 h (λ_{max} = 550 nm) and from a sample that was irradiated with a cutoff filter of $\lambda_{max} >$ 570 nm, yielding a mixture of H450 and H430 species. HPLC analysis indicated that all extracted chromophores consist exclusively of the original 13-*cis* locked retinal as is evident by comparing samples before and after irradiation (Fig. 4 *A*). To further investigate and verify the nature of the photoreaction product we carried out sodium borohydride reductions of the pigment and its various photointermediates. After illumination, this reaction reduces the chromophore-protein Schiff base bond and produces a retinal-protein stable covalent bond preventing chromophore extraction. The reduction reaction does not occur in the dark, therefore the retinal is extractable in the aldehyde form. In contrast, a retinal chromophore produced by photohydrolysis will be reduced to an extractable retinol. Therefore, this methodology can efficiently discriminate between a Schiff base linkage between the chromophore and the protein and an aldehyde hydrolysis product. Comparison of the reduction reactions carried out under dark and light conditions of pigment bR5.13 clearly indicated the presence of locked 13-*cis* retinol originating from aldehyde reduction (Fig. 4 *B*). This serves as a clear indication that irradiation induces an hydrolysis reaction. We note that the locked 13-*cis* retinol is much more polar than the locked 13-*cis* retinal. Therefore, we have used a more polar system for HPLC analysis. The various peaks were assigned by comparing them to synthetic chromophores (data not shown).


$$bR5.13 \xrightarrow{h\nu} H450 \xrightarrow{\Delta(\textit{several}\ \text{min})} H430 \xrightarrow{\Delta(\text{several hrs})} bR5.13$$

Scheme 2.

pH dependence of the hydrolysis reaction

The rate of the hydrolytic reaction was found to be affected by the pH, exhibiting more than a single component. Thus, under our characteristic illumination conditions, two components ($k_s = 1 \times 10^{-2}\ s^{-1}$; $k_f = 7 \times 10^{-2}\ s^{-1}$) were detected with rate constants independent of pH in the range 6 to 10. However, their relative amplitudes change significantly with pH with the k_f amplitude considerably increasing at high pH. As shown in Fig. 5, the relative fraction of the k_s component decreases at high pH. This behavior is best interpreted in terms of titration of a protein residue, characterized by pK_a = 8.2 (10 mM phosphate buffer; Fig. 5) that, in its deprotonated state, favors the fast hydrolysis path. Below pH 6, the hydrolysis is characterized by the same k_s component and by a second but different fast component (k_f^1), the rate of which is beyond our time resolution. The amplitude of the fast component (k_f^1) increases at low pH, and is characterized by pK_a = 4.15 (Fig. 5). Altogether, the fraction of the slow process (k_s) exhibits a bell-shaped behavior over the pH range 3 to 10. It is important to note that the low pK_a transition closely matches the purple-blue transition of pigment I (pK_a = 4; data not shown), which is well established to be due to the titration of Asp-85 (Metz et al., 1992). It is, therefore,

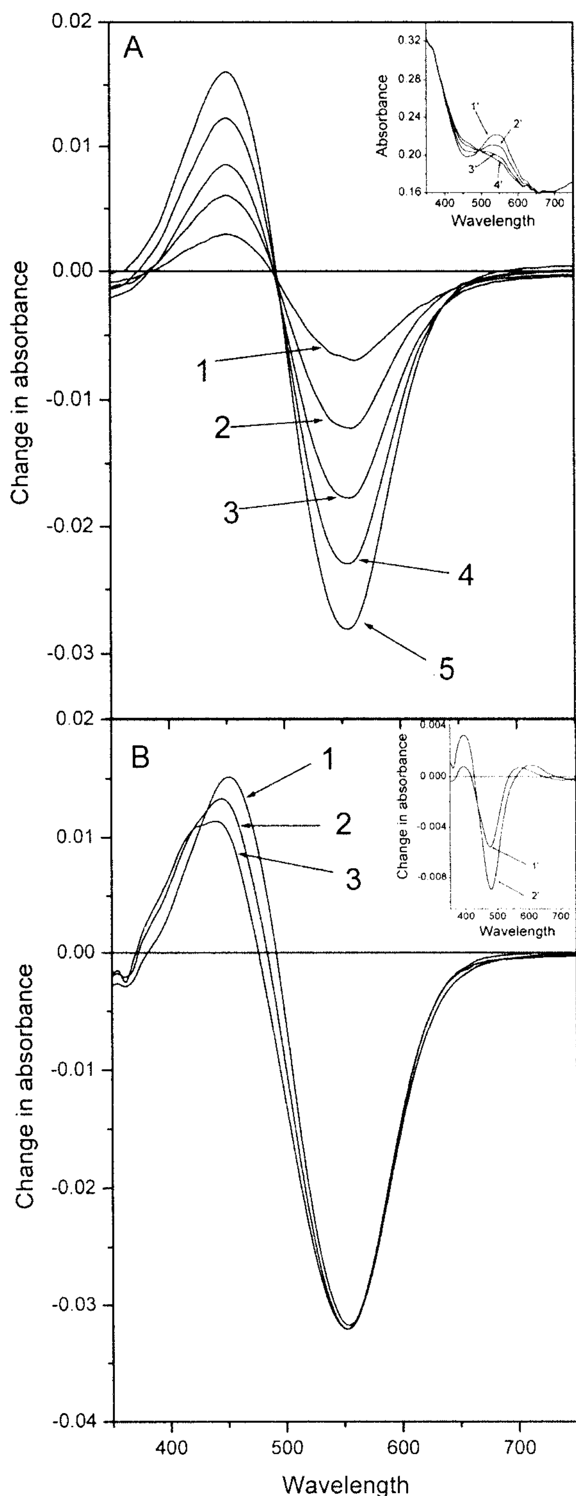


FIGURE 1 Irradiation of 13-*cis* locked pigment I at pH 9. (*A*) Difference absorption spectra at $t_i - t_0$, in which t_0 is the initial spectrum of the pigment before illumination. Spectra are shown after (*1*) 3 s, (*2*) 6 s, (*3*) 9 s, (*4*) 12 s, and (*5*) 15 s of irradiation. (*Inset*) Corresponding absorption spectra during the photoreaction. Spectra shown: 1′, initial spectra; 2′, 6 s; 3′,12 s; 4′, 15 s of irradiation. (*B*) Thermal decay of the 450-nm intermediate to the 430-nm intermediate after light-induced ($\lambda_{max} > 570$ nm) reaction. Irradiation was stopped after 80% of the pigment was bleached at pH 9. Difference absorption spectra at $t_I - t_{0,}$ in which t_0 is the initial spectrum of the pigment before irradiation. Spectra are shown (*1*) immediately after irradiation was stopped, (*2*) 50 s later, (*3*) 100 s after irradiation. (*Inset*) Difference absorption spectra showing the 450- to 430-nm transition. 1′, Difference between 2 and 1; 2′, difference between 3 and 1.

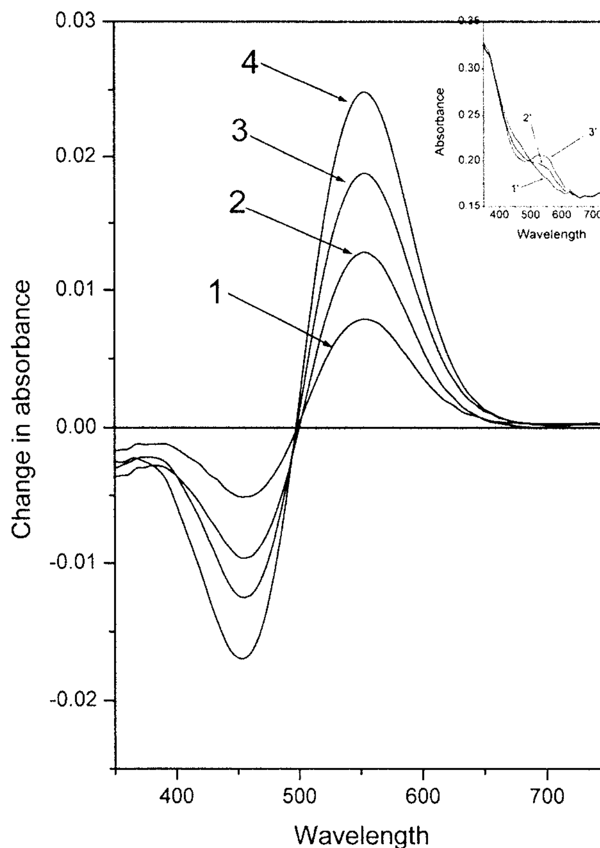


FIGURE 2 Blue light (420 nm < λ < 470 nm) rebinding induced by irradiation of the 450-nm intermediate after 30 s of pigment hydrolysis. Difference absorption spectra at $t_i - t_{0,}$ in which t_0 is the spectrum monitored after hydrolysis. Spectra are shown after (*1*) 4 s, (*2*) 8 s, (*3*) 25 s, (*4*) 120 s of blue light irradiation. (*Inset*) Corresponding absorption spectra during the rebinding reaction (1′) initial spectra, (2′) 4 s, and (3′) 30 s of irradiation.

plausible that the low pH dependence of k_s is associated with the titration of Asp-85. To support this suggestion, we have carried out the hydrolysis reaction in the D85N mutant reconstituted with chromophore **1**. The reaction rate was characterized by a single fast component (k_f^1) with $k_s \cong 0$ up to pH 7. This is consistent with the substitution of Asp-85 by a neutral residue that mimics the low pH protonation of Asp-85, as reflected by the purple to blue transition. Interestingly, analysis of the hydrolysis reaction of the Y185F mutant reconstituted with chromophore **1** reveals only one component (k_s) over the pH range 6 to 10 without any pH effect on the rate constant. We note that, unlike the native Y185F mutant, the reconstituted mutant with chromophore

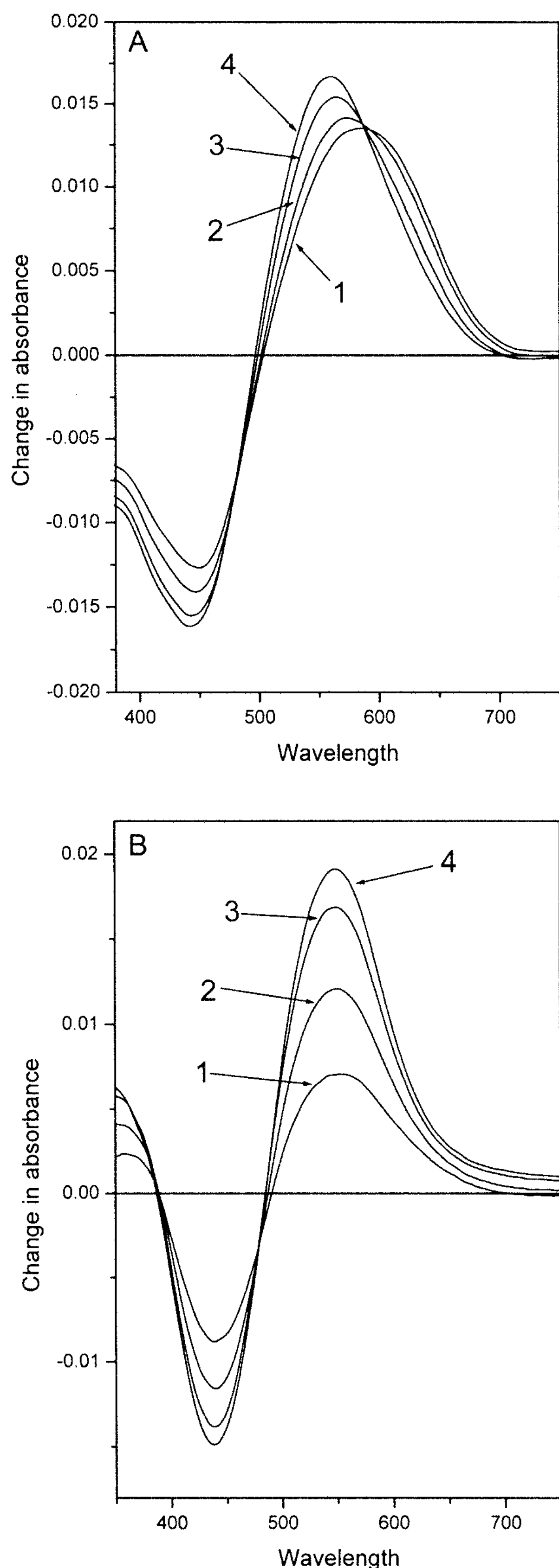


FIGURE 3 Difference spectra of blue light (420 nm < λ < 470 nm) rebinding induced by irradiation of the H430 intermediate, after thermal decay. (*A*) Illumination with blue light was carried out for 15 s at pH 7 after 800 s of light-induced pigment (λ > 570 nm) bleaching. Difference absorption spectra at $t_i - t_0$, in which t_0 is the last spectrum after hydrolysis. Spectra are shown after 15 s of blue light illumination (spectrum 1) followed by dark thermal decay [(spectrum 2), 30 s after pigment binding (spectrum 3) 60 s and (spectrum 4) 100 s]. (*B*) Blue light illumination at pH 9 after 200 s of hydrolysis. Difference absorption spectra at $t_i - t_0$, in which t_0 is the spectrum taken after photohydrolysis. Spectra are shown after (*1*) 30 s, (*2*) 60 s, (*3*) 100 s, and (*4*) 160 s after the photohydrolysis.

1 does not contain a detectable amount of species with protonated D85 in the pH range 6 to 10.

Protein conformational alterations

Unlike light-adapted bacteriorhodopsin (all-*trans* retinal), chromophore **1** is characterized by a 13-*cis* configuration. In addition, the retinal analog is bulkier relative to native retinal, since it bears a cyclopentene ring spanning carbons C_{13} and C_{14}. These two alterations should most probably affect the protein conformation relative to that of light-adapted bacteriorhodopsin. To study this possibility, we reconstituted 13-*cis* locked retinal **1** with the apomembrane of the A103C mutant followed by covalent binding of a nitroxide spin label to the 103 position. It was previously shown that the EPR spectrum of the nitroxide spin label at the 103 position of bacteriorhodopsin is sensitive to protein conformational alterations occurring in this region during the lifetime of the N photocycle intermediate (Thorgeirsson et al., 1997; Xiao et al., 2000). Since the N intermediate of the A103C-labeled mutant is characterized by a relatively long lifetime, it accumulates even under steady-state illumination, facilitating detection of alterations in the corresponding steady-state EPR spectrum (Fig. 6 *A*). Interestingly, as shown in Fig. 6 *B*, very similar alterations (relative to native bR) were observed in the artificial pigment of the A103C mutant derived from the 13-locked retinal 1 (Fig. 6 *B*). The EPR spectrum of this species was not affected by irradiation, consistent with its lacking of detectable photocycle. It is therefore suggested that incorporation of the 13-*cis*-locked chromophore into the binding site induces protein conformational alterations in the vicinity of Ala-103, which are similar to those observed in the N photochemically induced intermediate. In this respect, we note that our similar experiments with locked all-*trans* pigment (bR5.12) indicated that the locked all-*trans* chromophore did not induce protein conformational alterations in the vicinity of the Ala-103 residue. This experiment may indicate that the 13-*cis* configuration of bR5.13 is the major factor inducing the protein conformational alteration. However, because the additional rings in bR5.13 and bR5.12 are introduced at different regions of the retinal chromophore, it cannot be excluded that the bulky substitution affects the protein only in bR5.13.

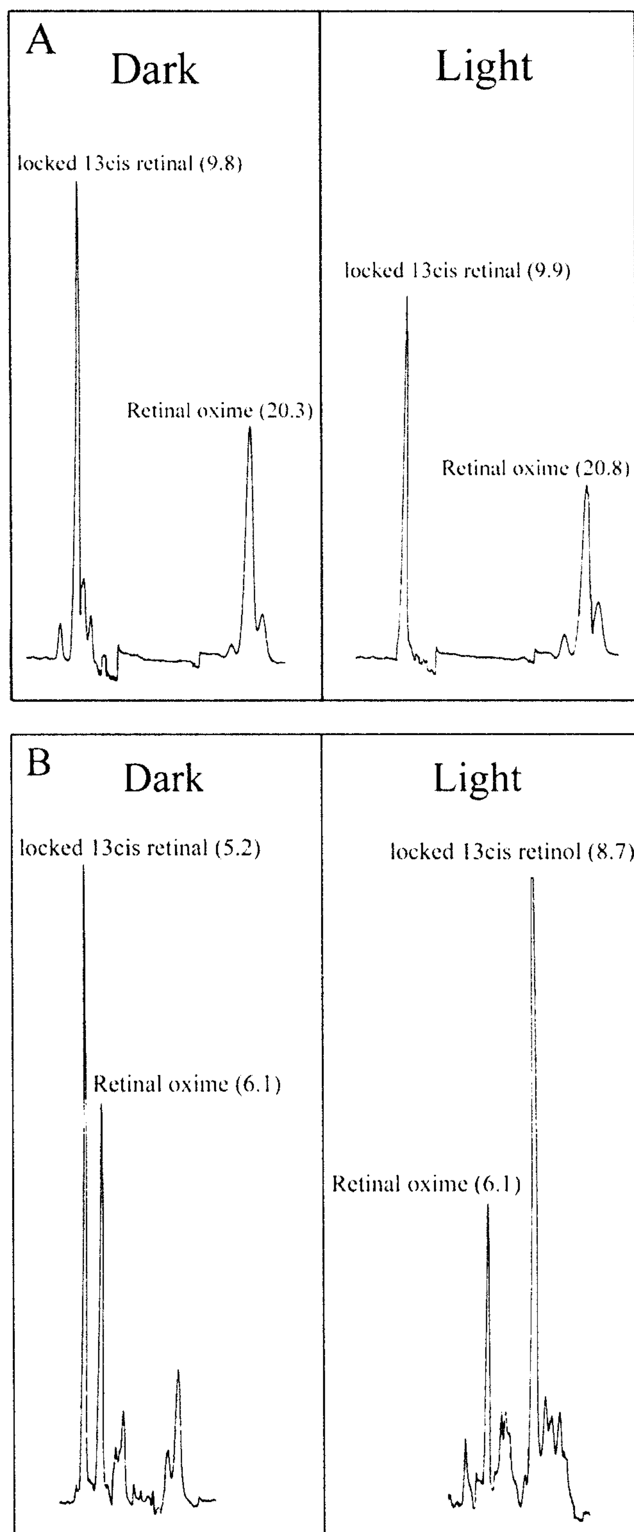


FIGURE 4 (*A*) HPLC analysis of the 13-*cis* locked retinal extracted from bR5.13 after incubation in the dark for 12 h and after light-induced bleaching. The extracted retinal analogs were eluted with hexane/ethyl acetate (90:10, v/v), numbers in parenthesis indicate elution time in minutes. (*B*) HPLC analysis (elution with hexane/ethyl-acetate 65:35, v/v) following reaction with sodium borohydride. (*Dark*) Extraction of the chromophores after incubation of the pigment with sodium borohydride in the dark for 30 min. (*Light*) Chromophore extraction after light-induced hydrolysis of bR5.13 in the presence of sodium borohydride.

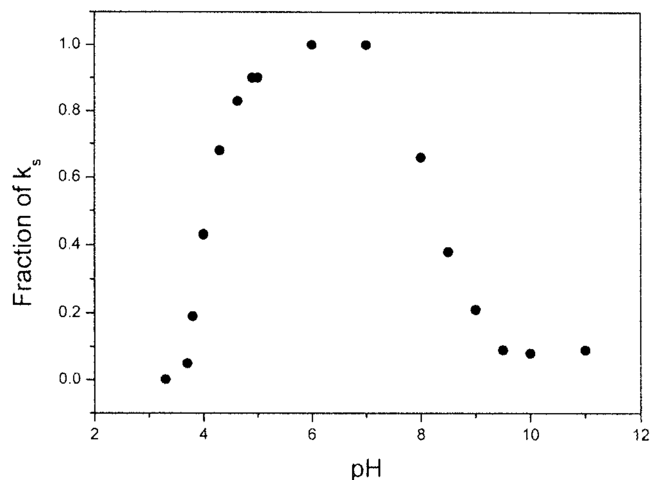


FIGURE 5 Rate of light-induced hydrolysis of bR5.13 as a function of pH. k_s is the slower rate constant of the hydrolysis reaction obtained by fitting the reaction trace to one and two components exponential decay (see Materials and Methods).

Hydrolysis reaction in the presence of osmotic active solutes

It was previously shown that the decay rate of the M intermediate in native bR decreases in the presence of osmotically active solute. These solutes probably effect bound water activity on the cytoplasmic side of the protein and decrease the proton transfer rate from Asp-96 to the retinal Schiff base (Cao et al., 1991). With the purpose of probing its sensitivity to water activity, we performed the hydrolysis reaction in the presence of osmotically active solutes, including 60% sucrose, 30% glucose, or 80% glycerol. Under identical illumination conditions, the slow reaction component (ks) was significantly reduced relative to that in water (1.8×10^{-1} s^{-1}; Table 1). Sucrose was the most effective additive, (1.55×10^{-3} s^{-1}), followed by glucose (1.7×10^{-3} s^{-1}), and glycerol (4.5×10^{-3} s^{-1}). The effect on the reaction rates was correlated with the known order of the solute osmotic effectiveness. To exclude the presence of viscosity effects on the reaction, we performed the hydrolysis in the presence of 5% POAC, which effects the viscosity but not the bound water in the protein. Indeed, the reaction rate (3.9×10^{-2} s^{-1}) was even faster than in water (1.8×10^{-2} s^{-1}), although the viscosity was significantly increased. The hydrolysis reaction was further studied by performing the reaction in a partially delipidated sample obtained by treatment with CHAPS. It has been shown (Jang and El-Sayed, 1988) that partial delipidization causes a reduction in the M decay rate, which is analogous

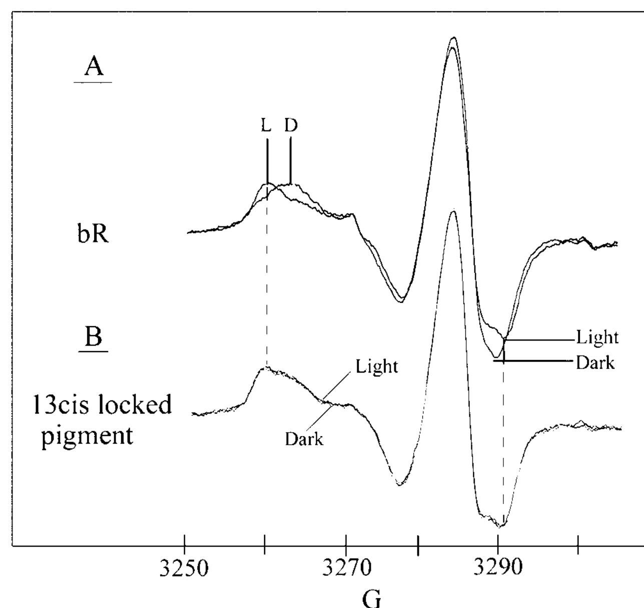


FIGURE 6 EPR spectra of spin-labeled bR and bR5.13 at 103 residue of mutant A103C in the dark and under light irradiation using 550-nm cutoff filter. (*A*) EPR spectra of spin-labeled bR. (*B*) EPR spectra of spin-labeled bR5.13.

to the effect of osmotic solutes. It was found that, under these conditions, the hydrolysis reaction rate of the bR5.13 pigment was significantly reduced (Table 1).

Locked 13-*cis* chromophore binding process to apoprotein

With the purpose of comparing the intermediates of the hydrolysis reaction with those of the retinal binding reaction, we incubated the locked *cis* chromophore **1** with the apo-protein. The 430-nm absorbing intermediate was formed in a rapidly established equilibrium (2–3 min). The pigment (bR5.13) was formed from this prepigment intermediate in a slow binding process over 5 to 6 h. As described above, a similar behavior was detected for the H430 hydrolysis product. Blue light irradiation at pH 7 of the 430-nm intermediate resulted in a fast binding reaction to produce a red-shifted pigment, absorbing at ~580 nm, which thermally decayed to the purple form (550 nm). This behavior bears close analogy to the blue light effect of the H430 hydrolysis intermediate. It is, thus, evident that the 430-nm prepigment bears a close resemblance to the H430 hydrolysis photoproduct.

TABLE 1 Reaction rate (ks) of the hydrolysis reaction in different solutes under identical illumination conditions

Solvent	Reaction rate ks (s^{-1})
60% Sucrose	1.55×10^{-3}
30% Glucose	1.7×10^{-3}
80% Glycerol	4.5×10^{-3}
5% POAC	3.9×10^{-2}
Water	1.8×10^{-2}

DISCUSSION

The efficient light-induced chromophore-protein Schiff base linkage hydrolysis detected in the present study is a unique property of the locked 13-*cis* pigment, bR5.13, in which the $C_{13}{=}C_{14}$ double bond isomerization is prevented. HPLC analysis indicates that the chromophores before and after the hydrolysis reaction are characterized by the same C=C configurations. The most plausible conclusion is that the pigment experienced light-induced hydrolysis without undergoing double bond isomerization. Alternatively, it may be argued that hydrolysis is triggered by a C=C or C=N photoisomerization, which is followed by a rapid thermal reversal to the original 13-*cis* configuration, after or before the hydrolysis. This possibility is highly unlikely but cannot be completely excluded.

The above conclusion, that double bond isomerization does not take place, is strongly supported by accumulated evidence showing that protein conformational alterations may be induced in artificial bacteriorhodopsin pigments in which isomerization about the *cis* or *trans* $C_{13}{=}C_{14}$ double bond is prevented by rigid ring structures (for a review, see Aharoni et al., 2001b). Such alterations were first detected in "locked" pigments by atomic force sensing of light-induced volume changes in bR membranes (Rousso et al., 1997a) and later using photothermal methods (Losi et al., 2000). Subsequently, compelling evidence in this respect was obtained by studying the light-induced cleavage of the protonated Sciff base bond of bR in the presence of hydroxylamine (Rousso et al., 1997b). Of special relevance are EPR studies monitoring the photocatalyzed oxidation and reduction reactions of a spin label covalently attached to selected protein residues (Aharoni et al., 2000). Thus, it was shown that the light induced reactivity is not restricted to the chromophore binding site and may extend to remote protein domains. In all cases the presence of light catalysis, even with "locked" bR, provided evidence for the occurrence of protein structural transformations that are not initiated by C=C isomerization. The most plausible rationalization of all of such observations is based on charge delocalization in the retinal excited state as the cause of the light-induced conformational changes in the protein (for a review, see Aharoni et al., 2001b). It was proposed that protein dipoles respond to the significant charge redistribution experienced by the retinal chromophore after light absorption. Reorientation of the protein dipoles induces conformational alterations, which may persist longer than the chromophore excited state lifetime. In support of this mechanism, it is a well-established fact that light excitation of bR initiates a large, induced electron redistribution in the polyene (Huang et al., 1989; Yamazaki et al., 1998).

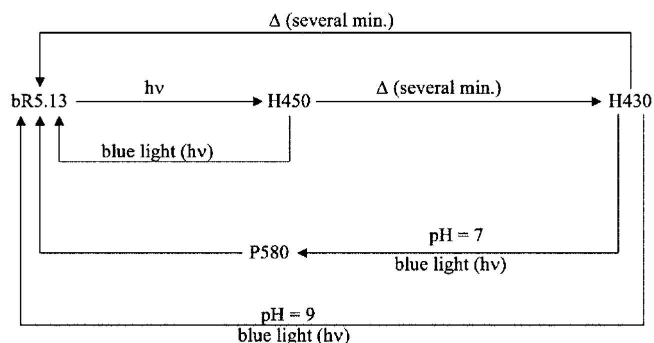
The present results indicate that an analogous mechanism may apply to the photohydrolysis of bR5.13. In this case the protein conformational alterations, induced by retinal light-induced charge delocalization, enable nucleophilic attack of water on the Schiff base linkage to form the free aldehyde. This reaction does not occur in the dark, although it is well established that water molecules are present in the binding site, including a water molecule that bridges the Schiff base, Asp-85 and Asp-212 (Luecke et al., 1999). As in the case of all previous photocatalytic effects in bR, we are still unable to define, on a molecular level, the overall changes in protein structure induced by light, as well as those responsible for any specific reaction. Nevertheless, in the present case, it is tempting to speculate that such changes allow for more water to penetrate into the retinal binding site and/or to induce reorganization of water molecules increasing the effective water concentration in the vicinity of the Schiff base bond. This mechanism is in keeping with the observed slowing down of the hydrolysis rate upon dehydration.

Irradiation with blue light (420 nm $< \lambda <$ 470 nm) after the hydrolysis process induces reformation of the chromophore-protein covalent bond. This phenomenon demonstrates that the protein experiences light induced conformational changes after light absorption by the retinal chromophore, which occupies the binding site without being covalently bound to the protein. The protein conformational alterations induce reformation of the protonated Schiff base, regenerating the original pigment. We note that these observations are in keeping with recent studies indicating that irradiation of a variety of molecules, which occupy the binding site without forming a chromophore-protein covalent bond, induces protein conformational alterations in the vicinity of residue 103 located on the cytoplasmic side of the protein (Aharoni et al., 2000, 2001a). Interestingly, in this case the blue light effect leads to rebinding, as compared with the opposite light catalyzed hydrolysis effect in the case of bR5.13. This apparent contradiction can be accounted for by recalling that in the two systems, the chromophores are located in different protein environments that stabilize the respective aldehyde and the protonated Schiff base (PSB) species. The suggested polarization effect perturbs, in each case, the specific protein structure, catalyzing the respective (hydrolysis or rebinding) process. Nevertheless the exact mechanism leading to such reactivity remains an open question. Of special interest will be to address the observation that both hydrolysis and rebinding are accelerated by light absorption by the respective "locked" chromophores.

The blue light effect can also serve as a tool to differentiate between intermediates in the hydrolysis process. Immediately after the hydrolysis reaction, the intermediate H450 is formed, which is reconverted very efficiently by blue light to the original pigment. The H450 species converts thermally to the subsequent intermediate H430, which responds differently to blue light. First it forms a red shifted intermediate (P580) absorbing similarly to the blue membrane of bR5.13 and is thus probably characterized by a protonated Asp-85 residue. This intermediate is thermally unstable and decays in few minutes at room temperature to the original bR5.13 pigment. The P580→bR5.13 thermal process is probably associated with protein conformational changes that decrease the Asp-85 pK_a and induces its deprotonation. At pH 8 the process leads to a mixture of P580 and of a 550-nm species. This observation can be accounted for in terms of an equilibrium between two protonation states of Asp-85, consistent with the pKa value of ~8 for Asp-85 in the photochemically induces intermediate. Scheme 3 describes the suggested pathway.

Previous studies have shown that light induced conformational changes take place at the cytolplasmic side of the membrane of artificial bR pigments in which $C_{13}{=}C_{14}$ isomerization was prevented by a rigid ring structure (Aharoni et al., 2000). Such conformational changes were detected by means of EPR spectroscopy that made use of the oxidation reaction of a reduced spin label attached to the protein at the 103 position. The present data suggest that such structural changes may be related to those that trigger the photohydrolysis reaction of bR5.13.

The hydrolysis reaction is highly sensitive to pH, being accelerated at high pH. Such acceleration is not observed in the Y185F mutant, which may suggest that deprotonation of Tyr-185 effects the hydrolysis reaction. Tyr-185 participates in the hydrogen-bonding network that prevails in the retinal binding site, and forms hydrogen bond with Asp-212. It is plausible that deprotonation of Tyr-185 disturbs the hydrogen bonding network accelerating the hydrolysis reaction. The hydrolysis is also significantly accelerated at low pH. This effect is associated with the purple to blue transition. It was demonstrated that this transition is due to protonation of Asp-85 that is part of the counter ion of the protonated Schiff base. The importance of the protonation state of Asp-85 in the hydrolysis reaction was further demonstrated using the D85N mutant in which Asp-85 residue is neutralized. The hydrolysis reaction in this mutant was significantly accelerated at pH 7 in keeping with the nega-



Scheme 3.

tive charge neutralization. The experiments demonstrate that the hydrogen-bonding network in the retinal binding site, which includes bound water controls the rate of the hydrolysis reaction.

The latter conclusion is further supported by the experiments with osmotically active solutes. These solutes create an osmotic pressure that withdraws water from the protein interior. It was previously shown that addition of osmotically active solutes to bacteriorhodopsin affects significantly the M intermediate decay time, which is controlled by proton transfer from Asp-96 to the retinal Schiff base (Cao et al., 1991). The proton transfer is probably mediated through a hydrogen-bonding network in the cytoplasmic part of the protein involving water molecules. It was observed that the M decay is correlated with the effectiveness of the osmotically active solute. A similar trend is observed in the hydrolysis reaction of bR5.13 in which the rate of the reaction was affected in the order of sucrose > glucose > glycerol in correlation with their molecular weight (342, 180, and 92, respectively). The decrease in reaction rate in the partially delipidated sample may further demonstrate the importance of water in the reaction. It was previously shown that a partially delipidated sample maintains the trimer structure of bacteriorhodopsin (Grigorieff et al., 1995) but decreases the rate of M decay (Jang and El-Sayed, 1988). One possibility is that the intact lipid structure is important for protein surface hydration, thereby affecting the water activity, especially at the cytoplasmic side of the protein. Bound water on this side of the protein is involved in proton transfer from Asp-96 to the retinal Schiff base reflected by the decay of the M intermediate. This suggests that water on the cytoplasmic side is an important component in the hydrolysis reaction and decreasing their activity, either osmotically or by partial delipidation, inhibits the hydrolysis reaction. However, another explanation for the decrease in the hydrolysis reaction rate in a partially delipidated sample is associated with possible protein coformational alteration induced by the delipidation process that affects the rate of the hydrolysis reaction.

In this respect, it is interesting to note that the EPR probe attached to 103C residue on the cytoplasmic side of bR5.13 resembles the EPR spectrum of the N intermediate rather than that of the wild type. It is tempting to suggest that bR5.13 adopts a conformation, at least on the cytoplasmic side in the vicinity of the 103 residue, which is similar to that of the N intermediate. We note that in both cases, the retinal chromophore adopts a 13-*cis* configuration. It was previously shown that the protein surface in the N intermediate is more hydrated than in the bacteriorhodopsin ground state (Hashimoto et al., 1998; Vonck, 2000). A similar situation may prevail in the 13-locked pigment, inducing its efficient hydrolysis reaction. A further analogy between bR5.13 and the N intermediate of native bR is associated with the observation that irradiation of native bR at high pH leads to a blue-shifted species, most probably due to an hydrolysis product (Balashov et al., 1991; Dancshazy et al., 1999). Since the N intermediate accumulates at high pH it may be the reason for the observed hydrolysis.

Thermal binding of the 13-*cis* locked aldehyde chromophore to the apomembrane is completed after 5 to 6 h and involves a 430-nm prepigment intermediate that is reminiscent of the H430 photohydrolysis. Blue light irradiation of the mixture obtained after 5 min of incubation resembles the behavior of H430 as well. It is most plausible that the 430-nm prepigment did not isomerize around any double bond during the thermal binding process. Therefore, the similar behavior of the H430 intermediate and the 430 nm prepigment further supports the conclusion derived from the HPLC analysis of H430 that this intermediate consists of a chromophore that adopts the configuration of 13-*cis* locked pigment **1**, and that pigment bR5.13 does not experience double bond isomerization during photohydrolysis. We suggest that the hydrolysis reaction proceeds via the reversed pathway of the binding process but allow trapping the 450-nm species (H450), which is not accumulated in the binding process. It was demonstrated previously that binding of native retinal to the apomembrane involves several intermediates absorbing between 430 and 470 nm, which retain the free aldehyde group (Schreckenbach et al., 1977; Schweiger et al., 1994). The red shift in the retinal aldehyde absorption induced by the protein is probably attributed to hydrogen bonding with the protein residues. The transformation to the pigment absorbing at 570 nm involves the formation of the protonated Schiff base linkage between the chromophore and the protein. Furthermore it was revealed that the covalent bond formation is prevented at low humidity (Rousso et al., 1995). It is tempting to suggest that similar intermediates prevail both in native retinal and 13-*cis* locked pigment-binding process, and the hydrolysis reaction reveals an intermediate that is not accumulated in the normal binding process and precedes the covalent bond formation.

Molecular dynamics calculations, which analyzed the hydrolysis process of the Schiff base linkage, have suggested that Lys-216 and the retinal form direct strong hydrogen bonding after the hydrolysis, which is broken by bound water in their vicinity by bridging the two residues thereby weakening their interaction (Isralewitz et al., 1997). It is possible that a similar process occurs in the hydrolysis of bR5.13. Nucleophilic attack of water on the Schiff base linkage will disconnect the chromophore-protein covalent bond and will produce retinal and free lysine, which may be bound by strong hydrogen bonding. The H450 intermediate may represent this situation and thus efficiently reconverts to the original pigment after blue light irradiation due to direct contact between the two residues. Light-induced protein conformational alteration leads to formation of a Schiff base bond. The proceeding intermediate (H430) may involve intervention of bound water molecules, which form a network of hydrogen bonding of the aldehyde group with

protein residues, causing the light-induced rebinding reaction to be less efficient.

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REFERENCES

Aharoni, A., B. Hou, N. Friedman, M. Ottolenghi, I. Rousso, S. Ruhman, M. Sheves, T. Ye, and Q. Zhang. 2001b. Non-isomerizable artificial pigments: implications for the primary light-induced events in bacteriorhodopsin. *Biochemistry (Moscow).* 66:1210–1219.

Aharoni, A., L. Weiner, A. Lewis, M. Ottolenghi, and M. Sheves. 2001a. Non-isomerizable non-retinal chromophores initiate light induced conformational alterations in bacterio-opsin. *J. Am. Chem. Soc.* 123: 6612–6616.

Aharoni, A., L. Weiner, M. Ottolenghi, and M. Sheves. 2000. Bacteriorhodopsin experiences light-induced conformational alterations in nonisomerizable $C_{13}{=}C_{14}$ pigments. *J. Biol. Chem.* 275:21010–21016.

Balashov, S., R. Govindjee, and T. Ebrey. 1991. Red shift of the purple membrane absorption bond and the deprotonation of tyrosine residues at high pH. *Biophys. J.* 60:479–490.

Cao, Y., G. Varo, M. Chang, B. Ni, R. Needleman, and J. Lanyi. 1991. Water is required for proton transfer from aspartic-96 to the bacteriorhodopsin Schiff base. *Biochemistry.* 30:10972–10979.

Dancshazy, Z., Z. Tokaji, and A. Der. 1999. Bleaching of bacteriorhodopsin by continuous light. *FEBS Lett.* 450:154–157.

Fischer, U., and D. Oesterhelt. 1980. Changes in the protonation state of bacterio-opsin during reconstitution of bacteriorhodopsin. *Biophys. J.* 31:139–145.

Grigorieff, N., E. Beckmann, and F. Zemlin. 1995. Lipid location in deoxycholate-treated purple membrane at 26 Å. *J. Mol. Biol.* 254: 404–415.

Hashimoto, S., M. Sasaki, and H. Takeuchi. 1998. Ultraviolet resonance Raman evidence for the opening of a water permeable channel in the M to N transition of bacteriorhodopsin. *J. Am. Chem. Soc.* 120:443–444.

Huang, J., Z. Chen, and A. Lewis. 1989. Second-harmonic generation in purple membrane-poly(vinyl alcohol) films: probing the dipolar characteristics of the bacteriorhodopsin chromophore in bR_{570} and M_{412}. *J. Phys. Chem.* 93:3314–3320.

Isralewitz, B., S. Izrailev, and K. Schulten. 1997. Binding pathway of retinal to bacterio-opsin: a prediction by molecular dynamics simulations. *Biophys. J.* 73:2972–2979.

Jang, D., and M. El-Sayed. 1988. Deprotonation of lipid-depleted bacteriorhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* 85:5918–5922.

Lanyi, J. 1999. Bacteriorhodopsin. *Int Rev Cytol.* 187:161–202.

Lewis, A. 1978. The molecular mechanism of excitation in visual transduction and bacteriorhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* 75: 549–553.

Losi, A., I. Michler, W. Gartner, and S. E. Braslavsky. 2000. Time resolved thermodynamic changes photoinduced in 5.12-*trans* locked bacteriorhodopsin. Evidence that retinal isomerization is required for protein activation. *Biophys. J.* 72:590–597.

Luecke, H., B. Schobert, H. T. Richter, J. P. Cartailler, and J. K. Lanyi. 1999. Structure of bacteriorhodopsin at 1.55 A resolution. *J Mol Biol.* 291:899–911.

Metz. G., F. Siebert, and M. Engelhard. 1992. Asp^{85} is the only internal aspartic acid that gels protonated in the M intermediateand the purple-to-blue transition of bacteriorhodopsin. *FEBS Lett.* 303:237–241.

Oesterhelt, D. 1998. The structure and mechanism of the family of retinal proteins from halophilic archaea. *Curr. Opin. Struct. Biol.* 8:489–500.

Oesterhelt, D., L. Schumann, and H. Gruber. 1974. Light-dependent reaction of bacteriorhodopsin with hydroxylamine in cell suspensions of *Halobacterium halobium*: demonstration of an apo-membrane. *FEBS Lett.* 44:257–261.

Ottolenghi, M., and M. Sheves, editors. 1995. Photophysics and photochemistry of retinal proteins. *Isr. J. Chem.* 35:193–513.

Rousso, I., I. Brodsky, A. Lewis, and M. Sheves. 1995. The role of water in retinal complexation to bacterio-opsin. *J. Biol. Chem.* 270: 13860–13868.

Rousso, I., N. Friedman, A. Lewis, and M. Sheves. 1997b. Evidence for a controlling role of water in producing the native bacteriorhodopsin structure. *Biophys. J.* 73:2081–2089.

Rousso, I., Y. Gat, A. Lewis, M. Sheves, and M. Ottolenghi. 1998. Effective light-induced hydroxylamine reactions occurs with $C_{13}{=}C_{14}$ nonisomerizable bacteriorhodopsin pigments. *Biophys. J.* 75:413–417.

Rousso, I., E. Khachatryan, Y. Gat, I. Brodsky, M. Ottolenghi, M. Sheves, and A. Lewis. 1997a. Microsecond atomic force sensing of protein conformational dynamics: implication for the primary events in bacteriorhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* 94:7937–7941.

Schreckenbach, T., B. Walckhoff, and D. Oesterhelt. 1977. Studies on the retinal-protein interaction in bacteriorhodopsin. *Eur. J. Biochem.* 76: 499–511.

Schweiger, U., J. Tittor, and D. Oesterhelt. 1994. Bacteriorhodopsin can function without a covalent linkage between retinal and protein. *Biochemistry.* 33:535–541.

Subramaniam, S., M. Lindahl, P. Bullough, A. Faruqi, J. Tittor, D. Oesterhelt, L. Brown, J. Lanyi, and R. Henderson. 1999. Protein conformational changes in the bacteriorhodopsin photocycle. *J. Mol. Biol.* 287: 145–161.

Szundi, I., and W. Stoeckenius. 1987. Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin. *Proc. Natl. Acad. Sci. U. S. A.* 84:3681–3684.

Thorgeirsson, T. E., W. Xiao, L. S. Brown, R. Needleman, J. K. Lanyi, and Y. K. Shin. 1997. Transient channel-opening in bacteriorhodopsin: an EPR study. *J Mol Biol.* 273:951–957.

Vonck, J. 2000. Structure of the bacteriorhodopsin mutant F219L N intermediate revealed by electron crystallography. *EMBO J.* 19:2152–2160.

Yamazaki, M., J. Goodisman, and R. Birge. 1998. Quadratic electrooptic effects in bacteriorhodopsin: measurements of gamma in dried gelatic thin films. *J. Chem. Phys.* 108:5876–5887.

Xiao, W., L. Brown, R. Needleman, J. Lanyi, and Y. Shin. 2000. Light-induced rotation of a transmembrane a-helix in bacteriorhodopsin. *J. Mol. Biol.* 304:715–721.