Nonisomerizable Non-Retinal Chromophores Initiate Light-Induced Conformational Alterations in Bacterioopsin

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Abstract: The photoactivation of retinal proteins is usually interpreted in terms of C═C photoisomerization of the retinal moiety, which triggers appropriate conformational changes in the protein. In this work several dye molecules, characterized by a completely rigid structure in which no double-bond isomerization is possible, were incorporated into the binding site of bacteriorhodopsin (bR). Using a light-induced chemical reaction of a labeled EPR probe, it was observed that specific conformational alterations in the protein are induced following light absorption by the dye molecules occupying the binding site. The exact nature of these changes and their relationship to those occurring in the bR photocycle are still unclear. Nevertheless, their occurrence proves that C═C or C═NH^+ isomerization is not a prerequisite for protein conformational changes in a retinal protein. More generally, we show that conformational changes, leading to changes in reactivity, may be induced in proteins by optical excitation of simple nonisomerizable dyes located in the macromolecular matrix.

Introduction

Bacteriorhodopsin (bR) is the integral protein of the purple membrane of *Halobacterium salinarum* and serves as a light-driven proton pump.[1] It is composed of seven transmembrane helices enclosing the binding pocket for an all-*trans* retinal chromophore, which is bound to Lys 216 via a protonated Schiff base (SBH^+). Absorption of a photon by the retinal eventually induces an all-*trans*→13-*cis* isomerization. This step is associated with the initial storage of a substantial fraction of the photon energy and results in several distinct intermediates characterized by an altered protein conformation.[2]

Early theoretical considerations advocated the induction of protein conformational changes by charge redistribution in the excited retinal chromophore.[3] Nevertheless, the predominant working hypothesis in retinal proteins has been that all protein changes are exclusively due to a *cis*→*trans* or *trans*→*cis* C═C, or C═N, isomerization of the retinal moiety. In other words, it is commonly postulated that *transient, light-induced, conformational changes in the protein can be induced only as a result of isomerization of the embedded chromophore around a "critical" double bond.*

This basic postulate has recently been questioned by a series of experiments indicating that light-induced conformational changes in the opsin (although not essentially related to those associated with the photocycle) are induced in artificial bR in which isomerization about the critical C_{13}═C_{14} bond was prevented by a rigid ring structure.[4,5] Morover, it was also shown that the covalent linkage to the protein is not required for inducing such alterations.[6] These findings advanced the more general concept that chromophore photoisomerization is not a prerequisite for the induction of conformational changes in the surrounding macromolecular matrix.

A difficulty with the above findings stemmed from the fact that all retinal analogues employed in these studies carried isomerizable double bonds other than the critical C_{13}═C_{14} bond. Thus, although highly unlikely, these could coincidentally replace the critical double bond that was specifically blocked. In other words, the data suggested but did not unambiguously prove that isomerization is not an essential prerequisite for the changes in protein conformation. In the present work we have circumvented the above difficulty by incorporating into the bacterioopsin binding site chromophores that carry no free isomerizable bonds. Since such a goal is extremely difficult to achieve with retinal analogues we have reverted to molecular dyes that bear no relationship to retinals. On one hand, this diminishes the analogy between the investigated systems and the natural retinal proteins. However, on the other hand, it substantially contributes to the generality of the effect.

The reported experiments definitively indicate that double bond isomerization is not a prerequisite for light-induced conformational alterations in the bacteriorhodopsin protein. More generally, they show that conformational changes in a macromolecule may be induced by excitation of an appropriately complexed, nonisomerizable, dye molecule.

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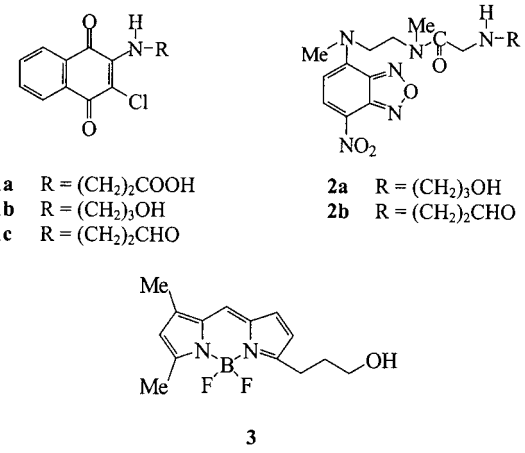
Scheme 1



1a R = $(CH_2)_2COOH$
1b R = $(CH_2)_3OH$
1c R = $(CH_2)_2CHO$

2a R = $(CH_2)_3OH$
2b R = $(CH_2)_2CHO$

3

Experimental Section

Sample Preparation. The spin-labeled (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSSL, TRC, Ontario, Canada) was covalently attached to cysteine residue of the appropriate bR mutant to yield the spin-labeled side chain. A solution of 10 μL of 100 mM MTSSL was added to 2 mL suspension of 50 μM bR mutant in 0.1 M phosphate buffer (pH 8) and 0.1 M NaCl. The suspension was 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% BSA. EPR measurements were used to estimate the extent of labeling and to ensure removal of nonbound spin label.

Dye Preparation. Naphthoquinone derivatives were prepared by condensation of 2,3-dichloro-1,4-naphthoquinone with β-alanine or aminopropanol to give **1a** and **1b** according to the previously described method.[7] Alcohol **1b** was further oxidized to aldehyde **1c** using pyridinium chlorochromate (PCC) in methylene chloride at room temperature for 1 h followed by chromatography in silica gel with ether mixed with 1% of methanol. 7-Nitrobenz-2-oxa-1,3-diazole derivative **2a** was prepared by condensation of iodo acetamide NBD (IANBD amide, Molecular Probes) with 3-aminopropanol in ethanol at room temperature for 12 h followed by silica gel chromatography to produce **2a**. Further oxidation of **2a** with pyridinium chlorochromate in methylene chloride at room temperature for 1 h gave aldehyde **2b** which was purified by chromatography with silica gel using ether as an eluent. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propanol (BODIPY FL propanol) was purchased from Molecular Probes.

Preparation of Spin-Labeled Bacterioopsin Dye Complex. Bacterioopsin of the appropriate spin-labeled bR mutant was prepared according to previously described method.[8] The appropriate dye (2.5 equiv) (Scheme 1) was added to spin-labeled apomembrane suspension at pH 7, and the mixture was incubated for 2 h at room temperature. The CD spectrum of the apomembrane−dye complex was compared to spin-labeled apomembrane spectrum without the dye. The extent of binding of the dye to the apomembrane binding site was monitored by the decrease in the retinal oxime positive peak at 360 nm. All CD measurements were performed on an AVIV circular dichroism spectrometer model 202.

For absorption maxima measurements the complexes were prepared by incubation of the apomembrane with 0.5 equiv of the dye to obtain almost complete binding.

Oxidation of the Spin-Labeled Apomembrane−Dye Complex. Reduction of the spin label was achieved during the bleaching procedure with hydroxylamine of the spin-labeled bR mutant to yield the reduced spin-labeled apomembrane as described previously.[6] After removal of the HA by four rounds of washing with water, the apomembrane was incubated with the dye to form the apomembrane−dye complex. The spontaneous oxidation by molecular oxygen was monitored by following the increase in the main peak of the EPR spectra (3288G for spin-labeled A103C mutant). Experiments were carried out in the dark or under illumination with a halogen lamp with an output of 150 W equipped with a heat absorbing filter and a 450 nm glass cut off filter. Illumination was kept steady for the whole experiment. A starting EPR signal prior to illumination was observed due to spontaneous oxidation of the spin label in the dark which takes place during the manipulation of the sample following the bleaching and reduction reactions before the EPR measurement (ca. 2 h).

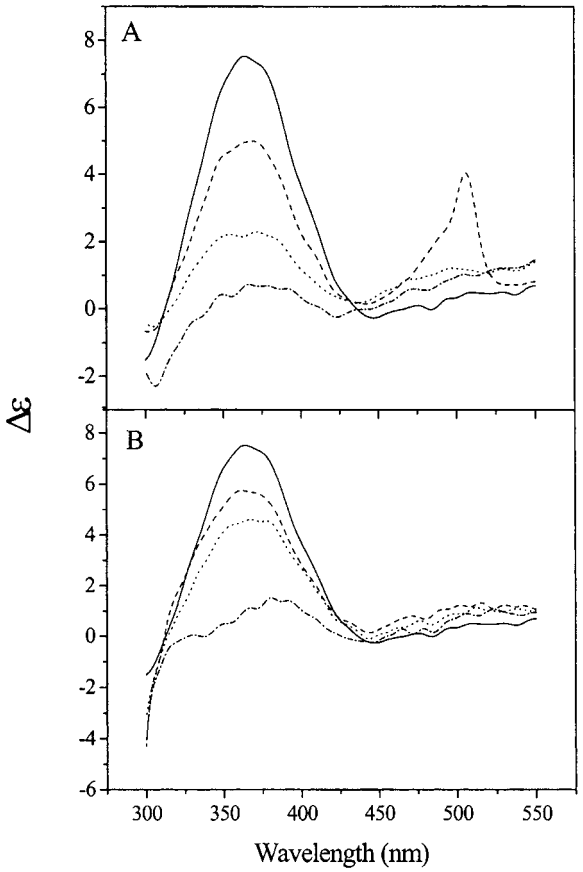


Figure 1. CD spectra of dye molecules incubated with bacterioopsin: (A) native bacterioopsin (—), incubation with bodipy **3** (- - -), NBD derivatives **2a** (···), and aldehyde **2b** (·-·-·-). (B) Native bacterioopsin (—), incubation with carboxylic acid **1a** (- - -), alcohol **1b** (···), and aldehyde **1c** (·-·-·-). All incubations were carried out with 2.5 equiv of the dyes for 2 h.

EPR measurements: All measurements were performed on a Bruker ER200 D-SRC spectrometer in a flat quartz cell (70 μL) at 295 K. No change in the line width of the EPR signal was observed during the oxidation reactions of the spin-labeled radical.

Results and Discussion

Incorporation of Dye Molecules into the Retinal Binding site. Several dye molecules characterized by a completely rigid structure in which no double bond isomerization is possible were incorporated into the bacterioopsin binding site (Scheme 1). This follows previous work[9] that had shown the ability of molecules characterized by appropriate functional groups and hydrogen-bond forming capabilities, to displace retinal oxime, which is the product of the bR bleaching procedure.[10] To prepare complexes with these molecules, bR was bleached by irradiation in the presence of hydroxylamine (HA). The resulting retinal oxime occupies the binding site as evidenced by a CD positive peak with a maximum at 360 nm (Figure 1). One indication of

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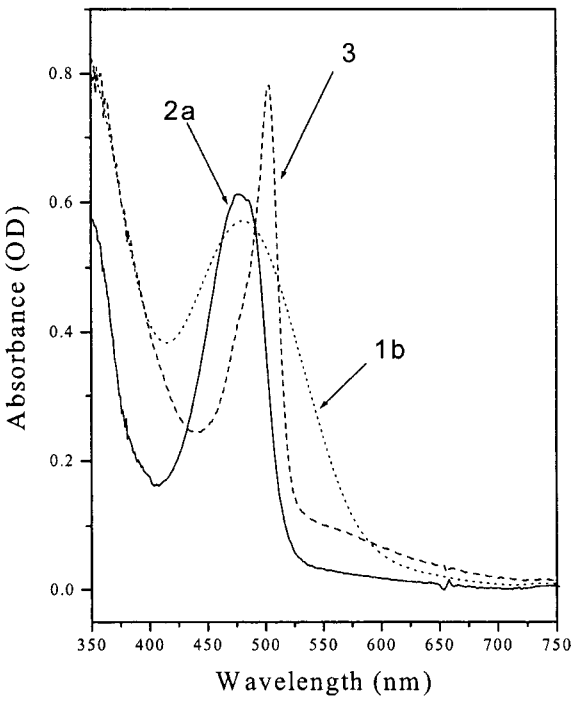

Figure 2. Absorption spectra of bacterioopsin−dye complexes.

the displacement of the retinal oxime from the binding site is a decrease in this positive peak.[11] Such a decrease is observed upon the addition of the dye molecules investigated in this paper (see Figure 1).

We have investigated the efficiency of several functional groups in displacing the retinal oxime (Figure 1). The fraction of binding site occupation by the dye can be estimated from the fraction of retinal oxime displacement. In the case of the naphthoquinone derivatives, **1a**−**1c** (Scheme 1), characterized by different functional groups, we observed different affinities to the binding site. The degree of binding increases in the order $RCOOH < RCH_2OH < RCHO$. A similar order was found for molecules, **2**, which bear hydroxy (**2a**) or aldehyde (**2b**) functional groups. Since the dyes that are shown in Scheme 1 differ in structure with respect to the retinal chromophore of bacteriorhodopsin, not all of them exhibit the CD spectrum that is normally seen in retinal proteins. In bacteriorhodopsin, such CD spectra occur, due to specific protein chromophore interactions[12] or dipole−dipole interaction between the retinal chromophores within the trimer structure of the protein.[13] Analogously to bR, one of these dye molecules, molecule **3**, does produce a distinct CD for the complex (Figure 1A), which further supports the experiments discussed above, indicating that these dyes occupy the regular retinal binding site. The absorption maximum of the apomembrane-dye complex of **3** is very similar to that of the dye absorption in solution (ETOH), whereas that of **1b** and **2a** are red-shifted by 13 nm (Figure 2), indicating that the protein does not effect significantly the absorption. The absorption maximum of native bacteriorhodopsin is considerably red-shifted relative to retinal protonated Schiff base in ethanol solution (570 vs 440 nm). This significant effect of the protein was attributed to mainly two factors: (1) Weak Schiff base-counterion electrostatic interactions prevails in the protein envirnoment relative to ethanol solution.[14] (2) An *s-trans* ring-chain planar conformation adopted by the retinal chromophore in the protein binding site as opposed to a twisted *s-cis* conformation in solution.[15] These two effects do not operate in the case of the apomemrane−dye complexes studied above, and therefore, it is not surprising that the absorption maxima of the complexes are similar to those measured in solution. Furthermore, the absorption maxima of dyes **1b, 2a**, and **3** are not shifted considerably in different solvents (Table 1). Therefore, it is not expected that the protein matrix itself would influence the absorption maxima significantly.

Table 1: Absorption Maxima of Dyes in Solution and Complexed with Apomembrane

	λ_{max} (nm)			
chromophore	hexane	CH_2Cl_2	EtOH	apomembrane
1b	459	471	472	485
2a	440	456	467	480
3	509	509	505	505

Detection of Light-Induced Conformational Changes by EPR Spectroscopy. We have recently developed a method based on EPR spectroscopy to detect light-induced conformational changes in bR pigments.[6] The method is based on following the oxidation of a reduced nitroxyl spin label attached to a site on the protein (other than the chromophore binding site) as a result of light absorption by a suitable retinal-based chromophore residing in the binding site. Specifically, we found that upon light absorption by a retinal oxime molecule that occupies the binding site, a significant acceleration of the radical oxidation by molecular oxygen occurs. The reaction was monitored by the appearance of the EPR signal of the oxidation product in the light, as compared to the same (thermal) reaction in the dark. The light-induced reaction indicates that the protein experiences conformational alterations following light absorption by the retinal oxime that increase the reactivity of the reduced radical toward molecular oxygen, probably due to increased oxygen accessibility to the label probe region.

In a similar fashion, to detect light-induced conformational changes in the present protein−dye complexes, we used the apoprotein mutant (A103C) that was chemically modified at the 103 position with the nitroxyl radical spin label. As shown in Figures 3−5 (presenting molecules **2a, 3**, and **1b)** we observed that the oxidation reaction under illumination of this spin label is significantly accelerated relative to the dark reaction in the case of all three molecules. The rates of light-induced oxidation were accelerated in the following fashion: bodipy (**3**), NBD amino propanol (**2a**), naphthoquinone amino propanol (**1b**) by 19.4, 4.2, and 1.7 times, respectively. We estimated the quantum yield of the light-induced reoxidation reaction of bacterioopsin **3** complex (ϕ_o). This was performed by carrying out experiments under the same illumination conditions of hydroxylamine bleaching reaction of bacteriorhodopsin in the presence of 1 M hydroxylamine at pH 7 (ϕ_h). A quantum yield

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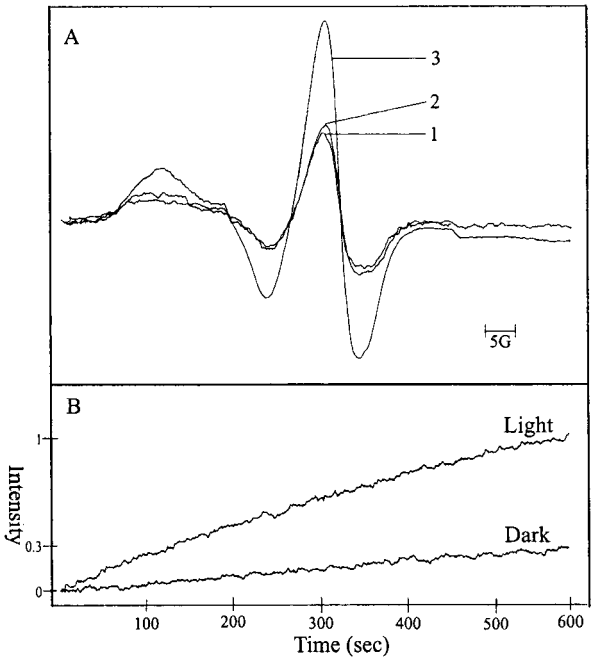

Figure 3. (A) EPR spectra of reoxidation by molecular oxygen of the bacterioopsin A103C spin-labeled mutant complexed with NBD amino propylaldehyde **2a**. Spectra of 1−2 were taken in the dark, whereas 3 was monitored following (λ > 450 nm) illumination at intervals of 10 min. (B). Kinetics of the reoxidation reaction under light and dark conditions, monitored at the central component of the EPR spectra of NBD amino propanol **2a**−bacterioopsin A103C complex.

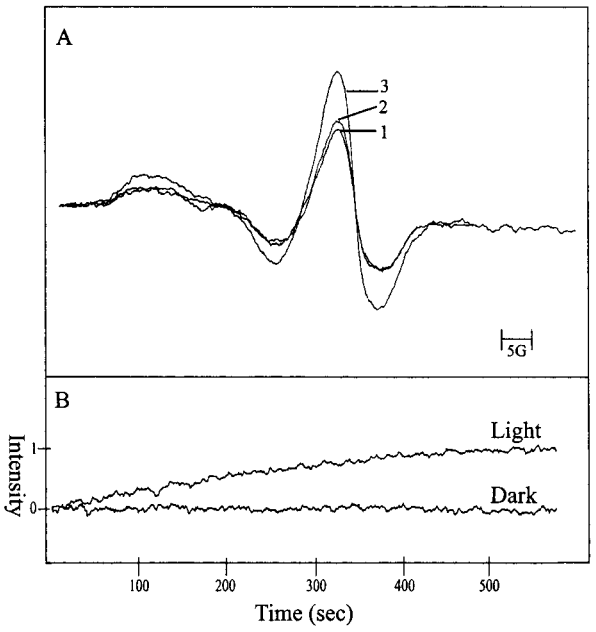

Figure 4. (A) EPR spectra of reoxidation by molecular oxygen of the bacterioopsin A103C spin-labeled mutant complexed with Bodipy **3**. Spectra of 1−2 were taken in the dark (with a 10 min interval between the two spectra), and 3 was monitored following illumination with a cutoff filter of λ > 450 nm for 700 s. (B). Kinetics of the reoxidation reaction under light and dark conditions, monitored at the central component of the EPR spectra.

ratio of $\phi_o = 0.8 \cdot \phi_h$ was obtained. Since it was previously found[5] that ϕ_h is on the order of 2×10^{-3}, we obtained $\phi_o \approx 1.6 \times 10^{-3}$.

We also investigated protein−dye spin-labeled complexes that employed mutant E74C. However, in this case no acceleration of the oxidation reaction with light was observed (Figure 6).

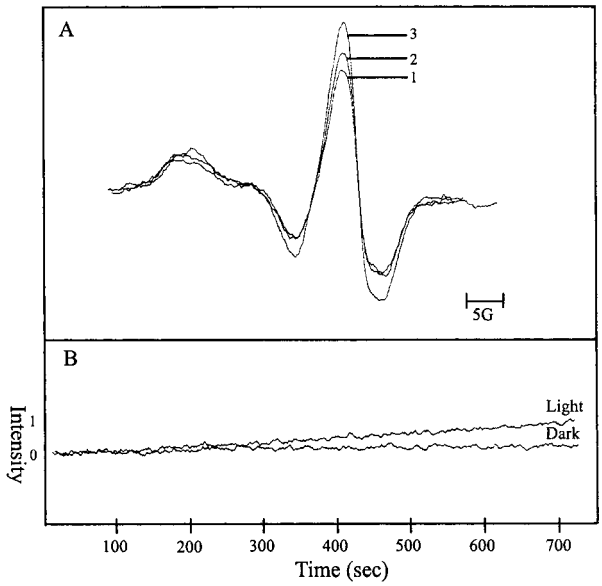

Figure 5. (A) EPR spectra of reoxidation by molecular oxygen of the bacterioopsin A103C spin-labeled mutant complexed with dye **1b**. EPR spectra 1 and 2 were taken in the dark at an interval of 10 min. Spectrum 3 was monitored following irridation for 11 min with a cutoff filter of λ > 450 nm. (B) Kinetics of the reoxidation reaction under light and dark conditions, monitored at the central component of the EPR spectra.

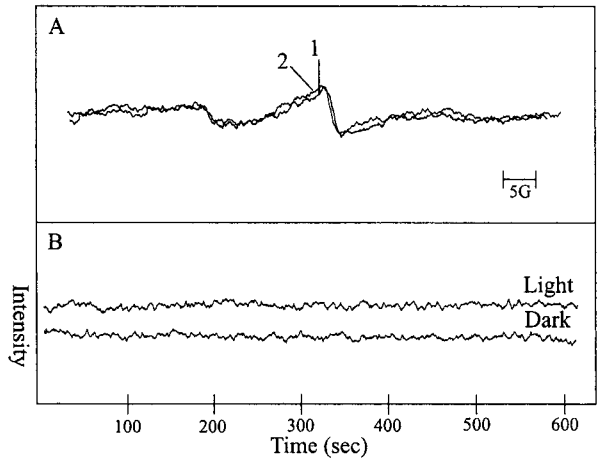

Figure 6. (A) EPR spectra of reoxidation by molecular oxygen of the bacterioopsin E74C spin-labeled mutant complexed with bodipy **3**. EPR spectrum 1 was monitored after incubation in the dark for 10 min, whereas 2 was monitored following illumination for 10 min with a cutoff filter of λ > 450 nm. (B) Kinetics of the reoxidation reaction of the bacterioopsin E74C spin-labeled mutant under light and dark illumination conditions. Illumination was carried out as described in (A).

This observation may point out at the specificity of the conformational changes induced by light around the 103 residue.

It is important to note that in all of the above experiments light absorption by the retinal oxime was prevented by irradiation with a 450 nm cutoff filter. We also point out that the irradiation did not cause any observable changes in the absorption spectra of the dye molecules, or in the CD spectrum of **3**, that could be indicative of any meaningful photochemical degradation. Moreover, to further confirm that the light-induced acceleration was due to light absorption by the dye occupying the binding site, we carried out several control experiments. First, we observed that irradiation of the spin-labeled apoprotein lacking the dye did not produce any oxidation acceleration following irradiation with a 450 nm cutoff filter. This is although

irradiation of the same sample below 400 nm resulted in the light-induced oxidation acceleration due to retinal oxime absorption (data not shown, see ref 6). To exclude the possibility that the light-induced reaction was due to nonspecifically associated dye molecules that are not in the binding site, we followed the oxidation reaction in a reduced A103C spin-labeled protein in which the protonated Schiff base linkage of the retinylidene chromophore was reduced by sodium borohydride. This produces a single covalent bond linking the chromophore to the protein and prevents displacement of the retinal chromophore from the binding site. Such a sample was incubated with the dye molecules, and the oxidation reaction was monitored by EPR. In this case, a light-induced reaction was not observed, in keeping with the incapability of the dye to occupy the binding site.

Analysis and conclusions. The results presented in the present work clearly indicate that light absorption by suitable dye molecules residing in the retinal binding site of bacteriorhodopsin leads to conformational changes in the A103 region of the protein. There are two main questions related to such changes that are still open. First, we are at present unable to speculate as to the molecular nature of such conformational changes, beyond pointing out that they appear to be specific to the A103 position, as compared, for example, to the E74 location. We also wish to emphasize that such changes are not essentially related to those occurring in the bR photocycle that are ultimately driving the cross-membrane proton transport. The latter most probably do require C_{13}═C_{14} isomerization, as indicated by the lack of a photocycle and any proton pumping activity in the case of C_{13}═C_{14} locked pigments.[16] Moreover, it is possible *but not essential* that the present changes detected by the EPR method bear a relationship to those observed in locked bR molecules, as detected by the methods described in refs 4−6, and recently also by photothermal methods.[17] Future work may shed light on the exact nature of the present EPR-monitored changes. A second aspect that is still undefined is the exact time course of the changes, namely, the time during which the structural transformation that catalyzes the probe oxidation persists. This cannot be revealed by the present continuous-excitation experiments where the rate of photoproduct accumulation is function of light intensity and quantum yield. Time scales, starting during the excited-state lifetime, extending into the millisecond range are all plausible and should be the subject of future work.

Nevertheless, despite lacking such exact definitions, the very existence of the light-induced phenomena leads to the conclusion that *optical excitation of a protein-embedded chromophore leads to conformational changes in the macromolecular host affecting chemical reactivity, which are not due to chromophore isomerization.*

The question arises as to the origin of these changes. The present results cannot exclude local thermal effects following light absorption by the chromophore. However, a more feasible suggestion for the origin of the protein conformational changes, which has previously been advanced for retinal proteins,[4−6] is charge redistribution developed in the different dyes following light absorption that triggers protein response, as previously advanced for the "locked" C_{13}═C_{14} artificial bacteriorhodopsin pigment. This mechanism was supported by experiments indicating that light-induced conformational changes are absent in a reduced pigment in which the polyene chain is symmetric, and therefore significant electronic charge redistribution, following light absorption, is highly unlikely. Upon the induction of a charge asymmetry in the reduced chain by appropriate substitution of the polyene, the light-induced conformational changes were restored.[4,6] Although a similar effect may also characterize the present dye systems, there is no evidence pointing at this direction. In fact, we still have no simple explanation for the difference in the photocatalytic efficiency of the various dyes that is lower, rather than higher, in molecule **1b** for which a higher degree of charge delocalization may be expected relative to **3** for example. Other factors, such as excited-state lifetime or molecular size and geometry (that may affect the exact positioning in the binding site) may be operative and effect charge redistribution in the chromophore following light absorption.

In conclusion, we have shown that conformational alterations in a protein, resulting in a change of chemical reactivity, may be triggered by optical excitation of a nonisomerizable molecule located in the binding site. In the case of the bR apoprotein, these alterations can be induced by a variety of molecules and are not limited to the basic structure and chemical properties of retinal. Most importantly, the light-induced changes in conformation are not due to C═C or C═NH^+ isomerization and do not require a chromophore−protein covalent bond. It is possible that analogous changes may also occur in the bR photocycle and may affect the proton-transport mechanism by coupling to changes induced by C═C isomerization. Such a relationship should be the subject of future work. More general applications of the present findings may be associated with triggering changes in protein function by optical excitation of a variety of dye molecules incorporated into appropriate binding sites.

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