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Light-Induced Charge Redistribution in the Retinal Chromophore Is Required for Initiating the Bacteriorhodopsin Photocycle

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Light absorption by a retinal isomer, bound to the protein via a protonated Schiff base linkage, triggers the characteristic photocycles of all retinal proteins, inducing the respective photobiological activity.[1] It has traditionally been assumed that such activity, for example, photosynthesis in the light-driven proton pump bacteriorhodopsin (bR), is exclusively due to protein structural changes caused by a primary isomerization of the retinal chromophore about a critical double bond, $C_{13}{=}C_{14}$ (*trans* to *cis*) in the case of bR. Unexpectedly, by using atomic force sensing[2a] and photothermal methods[2b] it was recently shown that the protein experiences conformational alterations even in artificial pigments in which the $C_{13}{=}C_{14}$ double bond isomerization is prevented by rigid ring structures. Moreover, accumulated evidence has recently indicated that several chemical reactions of the protein (other than those associated with the biologically active photocycle), which are due to protein conformational changes, may be photoinduced in bR even when isomerization of the $C_{13}{=}C_{14}$ (or any other C=C) bond is artificially precluded (see ref 2c for a review). Characteristic examples include: cleavage of the $C{=}NH^+$ bond by hydroxylamine,[2d] reduction of the same bond,[2e] and reduction−oxidation reactions of spin labels covalently bound to appropriate bR mutants.[2f,g]

On the basis of this evidence it was concluded that in all such cases the protein is activated via a mechanism that does not involve *trans* → *cis* isomerization. Protein activation was rather attributed to charge delocalization in the primarily generated excited state of the chromophore, in keeping with earlier suggestions based on theoretical considerations.[3a,b] Still, a major open question is the relevance of this novel mechanism to the photoactivity of retinal proteins in general and bR in particular. Specifically: Do the polarization-induced conformational changes couple in any way to those induced during the photocycle by *trans* → *13-cis* isomerization? Moreover, is the polarization effect a prerequisite for isomerization?

In the present work we approach these questions by studying several artificial bR pigments with a synthetic polyene designed so as to eliminate, or substantially reduce, the light-induced polarization of the chromophore. Both CF_3 substitution at and beyond the C_9 position (e.g., pigment **I** derived from chromophore **1**, Chart 1), and replacement of the β-ionone ring by an aromatic ring (pigments **II**, **III** derived from **2**, **3** respectively) are expected to inhibit positive charge delocalization to the ring end. To achieve further inhibition we prepared pigments derived from chromophores **4**, **5**, **6** that include *both* modifications. Since CH_3 replacement by CF_3 may also involve steric effects, control

Chart 1

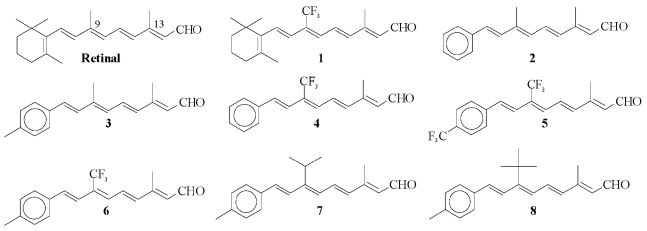

Table 1. Characterization of Native bR and Artificial Pigments

pigment	λ_{max} (nm)	SHG[a]	activity[b]	$k_1^L/k_1(obs)^D$ [c]	$k_2^L/k_2(obs)^D$ [c]
bR	568	1.0	(+)	40.8[d](1.0/0.0)	
I	520	0.375	(+)	3.83 (0.65/0.51)	22.3 (0.35/0.49)
II	500	0.75	(+)	32.3 (0.12/0.34)	13.9 (0.88/0.66)
III[e]	524	0.875	(+)	2.3 (1.0/0.34)	76.5 (1.0/0.66)
IV	450	−	(−)	0.4 (0.98/1.0)	
V	430	−	(−)	0.036 (0.75/0.80)	0 (0.25/0.20)
VI	462	−	(−)	0.62 (0.83/0.85)	0.09 (0.17/0.15)
VII	480	0.25	(+)	3.56 (0.98/0.95)	
VIII	430	−	(−)	−0.17 (0.69/0.49)	0.21 (0.31/0.51)

[a] Second harmonic generation signal relative to native bR. A dash implies that a signal could not be detected. [b] A pigment was deemed active (+) if photocycle intermediates were above the experimental detection limit, both at low temperatures and by pulsed-laser photolysis. [c] Kinetic data (in s^{-1}) for the reaction with hydroxylamine (see text for the definition of k^L and $k(obs)^D$). Numbers in parentheses indicate the fraction of each kinetic component in the dark/light. [d] Data are for 303 K, taken from ref 2d. [e] The dark reaction gave a two-exponential fit, while the light reaction gave only one. In this case the observed dark rates were each subtracted from the observed light rate.

experiments were also carried out with pigments **VII** and **VIII**, derived from **7** and **8**, carrying bulky isopropyl and *tert*-butyl groups at C_9, respectively.

The above artificial pigments (see Table 1 for their respective visible absorption maxima) were exposed to three sets of experiments: (a) Second harmonic generation (SHG) measurements, for probing the difference between ground- and (Franck−Condon) excited-state dipole moments.[4a,b] The experimental methodology used to obtain the SHG signal has been previously described.[4] We found that at a certain concentration, bR and its artificial pigments spontaneously form partially organized membrane fragments on a microscope cover slip. The extent of orientation of the film and the related SHG signal reached a plateau when increasing the concentration beyond an optical density of 0.1. This behavior allows for a comparison of the SHG signals of different pigments by using concentrations in the region of the plateau. The corresponding SHG signals for the molecules based on the chromophores of Chart 1, relative to that of bR, are given in Table 1. (b) Probing the occurrence of a photocycle by low-temperature continuous illumination and by room-temperature pulsed-laser photolysis. Characteristic

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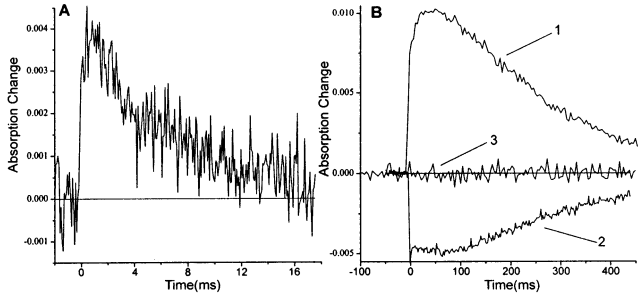

Figure 1. Laser-induced absorbance changes. **(**A**)** Pigment **II** monitored at 390 nm (M intermediate). **(**B**)** Pigment **II** monitored at 570 nm (1) and at 470 nm (2). Pigment **IV** monitored at 450 nm (3).

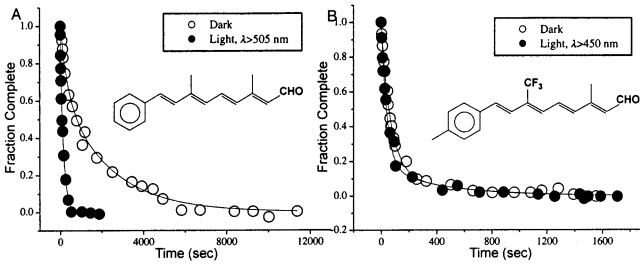

Figure 2. Bleaching of the visible band due to chromophore oxime formation monitored at 500 nm for pigment **II (**A**)** and at 460 nm for pigment **VI (B)** in the presence of 0.5 M hydroxylamine.

decay traces are shown in Figure 1. The presence (+) or absence (−) of an observable photocycle is reported in Table 1. (c) Occurrence of a light-induced reaction with hydroxylamine as followed by monitoring the bleaching of the main visible band.[2d] Figure 2 shows two characteristic cases. One (**II**) with a substantial light-catalysis effect, and one (**VI**) in which the decay under illumination coincides with that in the dark. The traces were best analyzed in terms of two exponential components. For each component the rate of the reaction was expressed by: $k(\text{obs})^{L} = k(\text{obs})^{D} + k^{L}$, where the two observed parameters represent the rate constant upon illumination and in the dark, respectively. k^{L} represents that of the light-induced reaction. The relative efficiency of the light-induced reaction for each decay component, estimated by the $k^{L}/k(\text{obs})^{D}$ ratio, is given in Table 1. We note that the origin of the two-component decay kinetics is not clearly established. One possibility is isomer heterogeneity attained under the photostationary conditions of our light-adapted membranes.[5] Alternatively, cooperativity effects[6] can also be operative. However, independently of the specific mechanism, it is evident that both components exhibit analogous chromophore-dependent trends.

First to be noted is the absence of a measurable SHG signal for all of the three 9-CF_3-substituted aromatic pigments **IV**, **V**, and **VI**. Molecules **I**, **II**, and **III** carrying only one of the two modifications, either the aromatic ring or 9-CF_3, exhibit (especially **I**) a detectable but substantially reduced SHG signal with respect to native bR. Of special interest is pigment **VIII** that, in variance with **2** and **3**, lacks a SHG signal probably due to a significant twist around the polyene single bonds imposed by the bulky substituent which prevents efficient charge delocalization especially in the excited state. The twist is also reflected in the blue-shifted absorption observed for pigment **VIII**. Significantly reduced SHG (relative to bR) is observed in pigment **VII** which bears an isopropyl substituent at C_9. The reduced SHG signal is in keeping with a twist in the polyene chain single bonds but a smaller one relative to pigment **VIII**.

Second to be considered is the occurrence of the photocycle. It is evident from Table 1 that photointermediates are observed with molecules **I**, **II**, **III**, and **VII**, which exhibit SHG signals, but *are beyond detection with molecules **IV**, **V**, **VI**, and **VIII** that lack a SHG signal*. In this respect it is worthwhile noting the uniqueness of the latter pigments in lacking a photocycle, despite maintaining the complete polyene chain and an uninhibited $C_{13}{=}C_{14}$ bond. We note that all of the chromophores derived from these pigments exhibit photoisomerization in solution (data not shown). An analogous conclusion may be derived for the light-catalyzed hydroxylamine reaction as reflected by the $k^{L}/k(\text{obs})^{D}$ ratio: molecules lacking a SHG effect (and a photocycle) exhibit negligible light acceleration, while substantial acceleration is observed in the case of SHG active (and photocycling) pigments.

The correlation between the occurrence of light-catalyzed reactions of the bR protein in artificial pigments and the presence of SHG activity has been previously observed[2d−g] and discussed.[2c] The findings of the present work are in keeping with this correlation but add information which is relevant to the very mechanism of bR function. Thus, it is evident that in the studied systems the *presence of a substantial light-induced charge redistribution in the retinal chromophore is a prerequisite for the occurrence of a photocycle* and thus of the related proton-pump activity. As previously suggested,[2a,c,f] the primary polarization of the chromophore induces a secondary polarization of its protein environment and consequently a transient change in protein structure. We now suggest that the protein polarization controls the course of the primary reaction by determining the relative efficiency of forward versus back-branching processes. If branching takes place in the excited state, a feasible possibility is one affecting the primary course of the reaction by catalyzing the transition from the *transoid* excited state to the partially isomerized conical intersection (CI) that channels the system back to the ground-state surface.[7] Alternatively, protein polarization may determine the yield and selectivity of the reaction, *after* crossing to the ground state at the CI point, by favoring the forward reaction to the completely isomerized *13-cis* photoproduct, over reversion to the initial *all-trans* state. While these conclusions are presently applicable to photosynthesis in bR, they may be effective in other retinal proteins including visual pigments.

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