Effective Light-Induced Hydroxylamine Reactions Occur with $C_{13} = C_{14}$ Nonisomerizable Bacteriorhodopsin Pigments

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ABSTRACT The light-driven proton pump bacteriorhodopsin (bR) undergoes a bleaching reaction with hydroxylamine in the dark, which is markedly catalyzed by light. The reaction involves cleavage of the (protonated) Schiff base bond, which links the retinyl chromophore to the protein. The catalytic light effect is currently attributed to the conformational changes associated with the photocycle of all-*trans* bR, which is responsible for its proton pump mechanism and is initiated by the all-*trans* → 13-*cis* isomerization. This hypothesis is now being tested in a series of experiments, at various temperatures, using three artificial bR molecules in which the essential C_{13}═C_{14} bond is locked by a rigid ring structure into an all-*trans* or 13-*cis* configuration. In all three cases we observe an enhancement of the reaction by light despite the fact that, because of locking of the C_{13}═C_{14} bond, these molecules do not exhibit a photocycle, or any proton-pump activity. An analysis of the rate parameters excludes the possibility that the light-catalyzed reaction takes place during the ~20-ps excited state lifetimes of the locked pigments. It is concluded that the reaction is associated with a relatively long-lived (μs-ms) light-induced conformational change that is not reflected by changes in the optical spectrum of the retinyl chromophore. It is plausible that analogous changes (coupled to those of the photocycle) are also operative in the cases of native bR and visual pigments. These conclusions are discussed in view of the light-induced conformational changes recently detected in native and artificial bR with an atomic force sensor.

INTRODUCTION

Retinal proteins, such as visual pigments and the light-driven proton pump bacteriorhodopsin (bR), consist of a retinal chromophore bound to the opsin via a protonated Schiff base with the ϵ-amino group of a lysine residue (for recent reviews see Ottolenghi and Sheves, 1995). The light-induced response of (vertebrate) visual rhodopsins leads to 11-*cis* → all-*trans* isomerization of the chromophore along with hydrolysis of the C═N bond. In contrast, the light response in all-*trans* bR is associated with C_{13}═C_{14} all-*trans* → 13-*cis* isomerization in a reversible photocycle, with no disruption of the C═N bond. However, the Schiff base in bR is susceptible to reaction with reagents such as hydroxylamine and sodium borohydride under illumination (Oesterhelt et al., 1973), and to a much smaller extent in the dark (Subramaniam et al., 1991).

The importance of the hydroxylamine reaction in bR in the dark, and primarily its enhancement by light, stems from its relevance to the structure of the retinal and of its protein environment, and to the conformational changes that both undergo during the photocycle. An ongoing working hypothesis has attributed the light-induced reaction to the M photointermediate of all-*trans* bR (Oesterhelt and Schumann, 1974; Oesterhelt et al., 1974), which is relatively long-lived and is known to be associated with deprotonation of the Schiff base and with substantial changes in protein structure (Ottolenghi and Sheves, 1995). This hypothesis has been seriously questioned by Subramaniam et al. (1991), who studied the reaction in a series of bR mutants solubilized in lipid/detergent micelles. No correlation was found between the efficiency of the light-induced reaction and the presence or absence (or the lifetime) of the M and subsequent N and/or O photointermediates. This led to the conclusion that the reaction occurs as a consequence of a conformational change caused by the light-catalyzed all-*trans* → 13-*cis* isomerization, but before deprotonation of the Schiff base at the M stage. The L precursor of M was suggested as the species that is reactive to hydroxylamine.

In the present work we adopt a different approach to the study of the light-induced hydroxylamine reaction in bR. The study is based on the artificial pigments $bR_{11,epoxy}$ (II), $bR_{5.12}$ (III), and $bR_{5.13}$ (IV) derived from the synthetic retinals (all-*trans*) 13-demethyl-11,14-epoxyretinal 2, all-*trans*-12,14-ethanoretinal 3, and 13-*cis*-13,14-propanoretinal 4, respectively (see Scheme I), in which the C_{13}═C_{14} *trans* → *cis* or *cis* → all-*trans* isomerization, which is a critical prerequisite of the photocycles of both all-*trans* and 13-*cis* bR, is prevented by an appropriate rigid ring structure. Early work by Bhattacharya et al. (1992a), who examined pigment III in lipid/detergent micelles, did not detect any light enhancement of the corresponding hydroxylamine reaction, as suggested by the absence of a photocycle. However, a study with an artificial visual rhodopsin with a locked C_{11}═C_{12} (*cis*) bond (Bhattacharya et al., 1992b) revealed a low-efficiency light-catalyzed hydroxylamine reaction, despite the lack of any photocycle. In the present work we have reexamined the reaction in bR by carrying out experiments in membrane suspensions of pigment III, over

Received for publication 12 February 1998 and in final form 9 April 1998.

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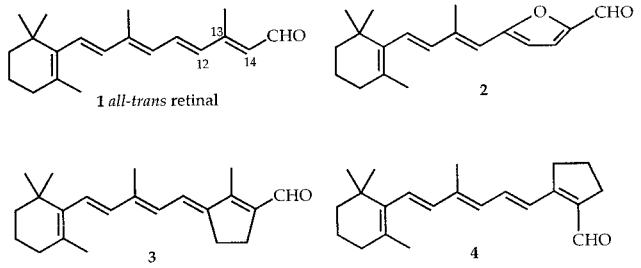
0006-3495/98/07/413/05 $2.00



Scheme 1

a range of temperatures, extending the work to an additional locked *trans* molecule (pigment II), as well as to the 13-*cis* locked pigment IV. Surprisingly, we find that effective hydroxylamine reactions are observed with all three synthetic pigments. An analysis of the kinetic parameters leads to the interpretation that the reactions are caused by light-induced conformational changes, extending to a μs-ms time scale, which are not associated with an optically detectable photocycle. It is plausible that analogous changes are also relevant to the same reaction in native bR, and possibly in visual pigments.

MATERIALS AND METHODS

Retinal analogs were prepared as previously described (Fang et al., 1983; Sheves et al., 1985). Bacteriorhodopsin was isolated according to the method of Oesterhelt and Stoeckenius (1974). Artificial pigments were prepared by incubating the analogs at 25°C with the apomembrane at pH 6.8. The bleaching reaction of bR with hydroxylamine was followed by monitoring the disappearance of the main absorption band (570 nm for II and III and 550 nm for IV). Experiments were carried out in the dark or under illumination, using 5 μM bR suspensions in the presence of 1 M hydroxylamine at pH 7, adjusted with NaOH and HCl. A thermostatted reaction cell in a HP 8453 spectrophotometer was employed. Illumination was carried out with a halogen lamp with an output of 150 W and equipped with a heat-absorbing filter and a 520-nm glass cutoff filter. Illumination was kept steady for the full set of experiments, and it was confirmed, by continuously monitoring the temperature of the solution by inserting a thermocouple into the solution, that it did not affect the thermostatted sample temperature. The temperature fluctuated by ± 0.5°C.

RESULTS AND DISCUSSION

Fig. 1 shows the spectral changes associated with the dark- and light-induced reactions with hydroxylamine of pigments I–IV. Analogous but considerably slower reactions also take place in the dark. All reactions are well represented by single exponentials, in keeping with those of native bR and of bR mutants (Oesterhelt et al., 1973, 1974; Oesterhelt and Schumann, 1974; Subramaniam et al., 1991). The experimental measured rate constants, as a function of temperature, in both light (k^l(ex)) and dark (k^d), are summarized in Table 1. We represent the dark reaction rate parameters as $k^d = A^d \exp(-E_a^d/RT)$. The light-catalyzed process was analyzed according to $k^l(\text{obs}) = k^l(\text{ex}) - k^d$, where k^l(ex) is the experimental rate constant in the light, which is the sum of the contributions of the dark reaction (k^d) and of the "observed" light-catalyzed reaction (k^l(obs)). k^l(obs) was calculated by extrapolating the appropriate k^d values. Arrhenius plots of the rate constants are given in Fig. 2, showing good fits to the expressions $k^d = A^d \exp(-E_a^d/RT)$ and $k^l(\text{obs}) = A^l(\text{obs}) \exp(-E_a^l/RT)$. The corresponding rate parameters are summarized in Table 2. Note that k^l(obs) and A^l(obs) are related to the intrinsic parameters of the light-induced reaction, k^l and A^l, by $k^l(\text{obs}) = k^l \cdot C$ and $A^l(\text{obs}) = A^l \cdot C$, where C is an undetermined function of the light intensity and of the quantum yield of the reaction. Evidently, $k^l(\text{obs}) \simeq k^l(\text{ex})$ when $k^l(\text{ex}) \gg k^d$, which is the case for pigments I and IV.

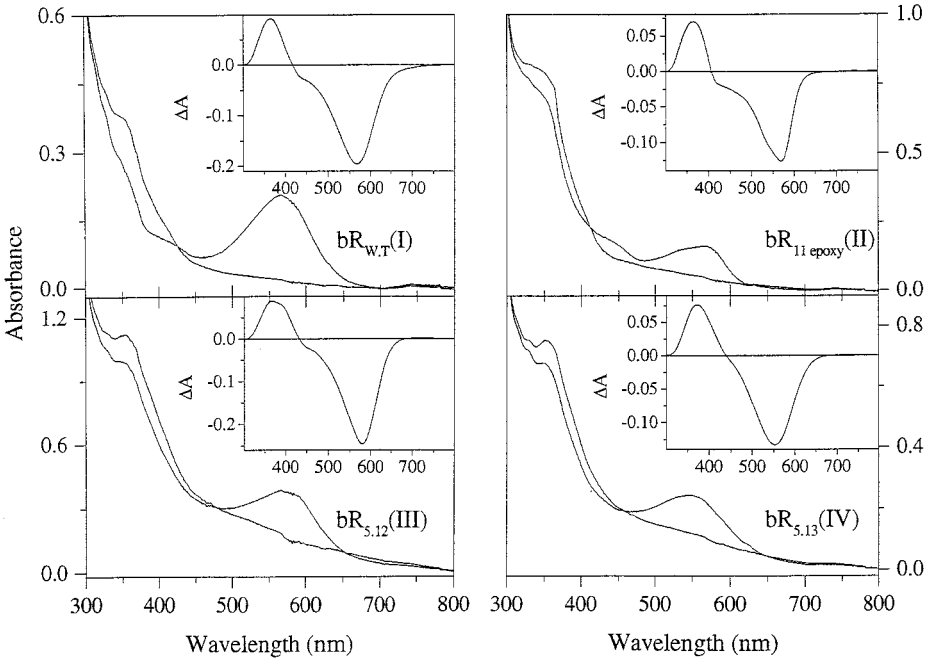


FIGURE 1 Absorption spectra of pigments I–IV, before and after exposure to illumination with a 520-nm cutoff filter in the presence of hydroxylamine at 298°K. Illumination was carried out until no detectable change was observed at 570 nm. (*Insets*) Corresponding difference spectra slowing bleaching of the visible band and formation of the UV band due to the retinal oxime photoproduct.

TABLE 1 Rate constants for measured light and dark hydroxylamine reactions of native bR(I) and artificial pigments II–IV

Temperature	WT(I)		bR_{11}, epoxy (II)		$bR_{5.12}$ (III)		$bR_{5.13}$ (IV)	
(K)	k^l(ex) (s^{-1})	k^d (s^{-1})	k^l(ex) (s^{-1})	k^d (s^{-1})	k^l(ex) (s^{-1})	k^d (s^{-1})	k^l(ex) (s^{-1})	k^d (s^{-1})
283							1.44×10^{-3}	2.72×10^{-5}*
288					1.78×10^{-3}	5.10×10^{-4}*	2.36×10^{-3}	4.39×10^{-5}*
293					2.20×10^{-3}	6.47×10^{-4}*	3.65×10^{-3}	6.96×10^{-5}*
298			3.01×10^{-3}	2.12×10^{-3}*	2.67×10^{-3}	8.22×10^{-4}*	6.48×10^{-3}	1.09×10^{-4}*
303	1.78×10^{-3}	4.26×10^{-5}*	3.85×10^{-3}	2.47×10^{-3}*	3.30×10^{-3}	1.00×10^{-3}*	11.95×10^{-3}	1.67×10^{-4}*
308	2.04×10^{-3}	6.65×10^{-5}*	4.62×10^{-3}	2.88×10^{-3}*	4.08×10^{-3}	1.20×10^{-3}*	19.25×10^{-3}	2.54×10^{-4}*
313	2.31×10^{-3}	1.02×10^{-4}*	5.64×10^{-3}	3.11×10^{-3}	6.03×10^{-3}	1.65×10^{-3}		
318	2.83×10^{-3}	3.03×10^{-4}	7.49×10^{-3}	3.73×10^{-3}	7.45×10^{-3}	1.82×10^{-3}		5.08×10^{-4}
323	3.47×10^{-3}	4.81×10^{-4}	8.35×10^{-3}	4.33×10^{-3}		2.20×10^{-3}		8.62×10^{-4}
328	3.85×10^{-3}	6.23×10^{-4}		4.88×10^{-3}		2.95×10^{-3}		10.90×10^{-4}
333		9.54×10^{-3}						1.60×10^{-4}

*Extrapolated from Arrhenius plot of the measured rate constants.

However, a substantial correction for the contribution of the dark reaction was needed for pigments II and III.

The following features are clearly evident:

1. Unexpectedly, both dark- and light-induced hydroxylamine reactions are observed with all three locked molecules. Moreover, in some of the cases such processes are as (or even more) effective than that of the native pigment. The catalytic effect of light in the case of the locked pigments is due to an increase in the frequency factor. In native bR, the reaction is light-accelerated because of a significant reduction in E_a, despite a significant decrease in the relative frequency factor.

2. The dark reaction rate for the locked *cis* pigment (IV) is similar to that of the native pigment, with respect to both frequency factor and activation energy. The reactions of pigments II and III are faster by factors of 10–30. This is due to a considerably lower E_a value, which more than compensates for the much lower frequency factor.

3. A different situation prevails in the case of the light-induced reactions. For all locked pigments $E_a^l > E_a^d$, whereas for native bR $E_a^l \ll E_a^d$.

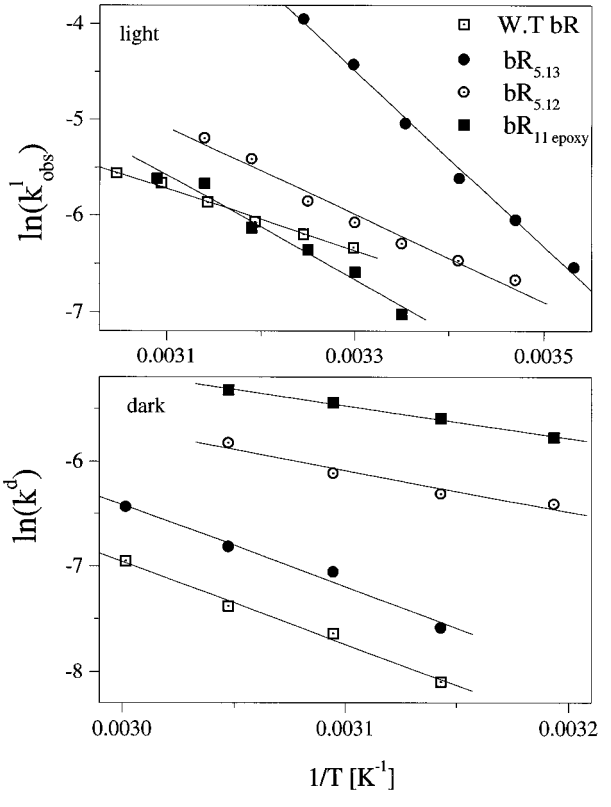


FIGURE 2 Arrhenius plots of the first-order rate constants of the hydroxylamine reactions of pigments I–IV in the dark (k^d) and under illumination ($k^l_{(obs)}$). Note that $k^l_{(obs)}$ represents the net contribution of the light-induced reaction after correction for that of the dark reaction.

We first address the key observation, namely, that light catalyzes the hydroxylamine reaction, despite the failure to detect a photocycle. Flash photolysis studies with time resolution in the >ns time range did not detect a photocycle in the locked pigments (Sheves et al., 1985; Chang et al., 1985; Bhattacharya et al., 1992a; Delaney et al., 1995). In addition, for pigment III, no accumulation of any photointermediate was observed at liquid nitrogen temperature by either absorption or Fourier transform infrared spectroscopies (Chang et al., 1985). A possible interpretation of these phenomena is that the reaction takes place in the excited state, which in the case of pigments III and IV has been shown to exhibit lifetimes of ~20 ps (Delaney et al., 1995; Zhang et al., 1996; unpublished results from our laboratory). We address this mechanism by estimating the quantum yield of the light-induced hydroxylamine reaction (ϕ_h) and by setting an upper limit to its rate constant. ϕ_h was determined by using the quantum yield of the 13-*cis* → all-*trans* (dark to light adaptation) reaction of native bR (ϕ_{dl}) for actinometry. This was performed by carrying out experiments, under the same illumination conditions, in which the rate of dark to light adaptation (monitored by the characteristic absorbance change at 600 nm) was compared to that of the hydroxylamine bleaching reaction. A quantum yield ratio of $\phi_{dl} \simeq 40\phi_h$ was obtained. Because ϕ_{dl} is on the order of 0.1 (Korenstein and Hess, 1977; Kalisky et al., 1977), we obtain for native bR $\phi_h \simeq 2 \times 10^{-3}$. Similar or even higher values characterize the locked pigments (see Table 2). This value of ϕ_h clearly excludes an excited state

TABLE 2 Rate parameters associated with the dark- and light (obs.)-catalyzed hydroxylamine reactions of native bR(l) and of the artificial pigments II–IV

	Dark				Light						
	k^{d}* (s^{-1}) (298°K)	$\tau^{d}_{1/2}$ (s) (298°K)	E^{d}_{a} (kcal/mol)	A^{d} (s^{-1}) (298°K)	$k^{l}_{(obs)}$ (s^{-1}) (298°K)	$\tau^{l}_{1/2}$ (obs) (s) (298°K)	E^{l}_{a} (kcal/mol)	$A^{l}_{(obs)}$ (s^{-1})	k^{l} (s^{-1}) (298°K)	A^{l} (s^{-1})	ϕ_h
I bR (native)	6.6×10^{-5}	10,400	15.5	1.5×10^{7}	1.4×10^{-3}*	495	6.4	7.0×10^{1}	—	—	$\sim 2 \times 10^{-3}$
II $bR_{11,epoxy}$	2.1×10^{-3}	330	6.1	6.2×10^{1}	8.9×10^{-4}	778	10.8	7.9×10^{4}	<10	$<10^{8}$	$\sim 2 \times 10^{-3}$
III $bR_{5.12}$	8.2×10^{-4}	846	7.9	5.0×10^{2}	1.8×10^{-3}	375	9.1	8.4×10^{3}	<10	$<10^{8}$	$\sim 2.5 \times 10^{-3}$
IV $bR_{5.13}$	1.1×10^{-4}	6,300	15.5	2.5×10^{7}	6.9×10^{-3}	100	18.2	1.5×10^{11}	$<10^{2}$	$<10^{15}$	$\sim 10^{-2}$

*Extrapolated from Arrhenius plot of the measured rate constants.

reaction in the case of the locked 13-*cis* pigment (IV). Thus, assuming an upper limit of $A^{l} \approx 10^{15}$ s^{-1}, the observed E^{l}_{a} value of 18 kcal/mol sets an upper limit for k^{l}, which at 298°K is on the order of 10^{2} s^{-1}. This is nine orders of magnitude slower than the excited state decay time, implying that for an excited state reaction, $\phi_h < 10^{-9}$. This value is highly inconsistent with the experimental value of $\sim 2 \times 10^{-3}$. The application of similar considerations to pigments II and III, for which $E^{l}_{a} = 9$–10 kcal/mol, sets an upper limit of $k^{l} \approx 10^{8}$ s^{-1} (298°K), and therefore $\phi_h < 5 \times 10^{-2}$. This value, which is based on the upper limit of $A^{l} \approx 10^{15}$ s^{-1}, is not inconsistent with the experimental value of $\phi \approx 2 \times 10^{-3}$. However, it appears that for pigments II and III, A^{l} is much smaller than 10^{15} s^{-1}. Thus, on the basis of the value of k^{l}(obs) = 1.5×10^{11} s^{-1}, reported in Table 2 for pigment IV, and because $A < 10^{15}$ s^{-1}, we can estimate that $C \leq 10^{-4}$. Thus, for pigments II and III, $A^{l} \leq 10^{8}$ s^{-1} and $k^{l} < 10$. According to the above discussion, this excludes an excited state mechanism for pigments II and III as well.

Excluding an excited state process, and on the basis of the above kinetic considerations, we are led to the conclusion that the reaction of the $C_{13}{=}C_{14}$ locked molecules takes place after relaxation to a ground state photoproduct, on a time scale that is longer than microseconds. As mentioned above, there is no spectroscopic evidence for a >ns photo-transformation of such molecules, and it has generally been assumed that the original ground state is repopulated within ~20 ps after excitation (Delaney et al., 1995; Zhang et al., 1996). Our data are thus at variance with this assumption, suggesting that a relatively long-lived structural change is induced in these locked bR pigments that catalyzes the hydroxylamine reaction. One way to account for this conclusion is to assume the presence of a low-yield ($\sim 10^{-3}$) photocycle in the case of the locked molecules (e.g., by isomerization around a C=C bond, other than $C_{13}{=}C_{14}$), which leads to the hydroxylamine reaction with a quantum yield, which is close to unity. However, this mechanism is highly unlikely, in view of the failure to observe accumulation of any photointermediates after light absorption at liquid nitrogen temperature in pigment III (Chang et al., 1985). In fact, independently of its primary quantum yield, there is no precedent for any retinal-protein photocycle whose primary photointermediates cannot be accumulated at cryogenic temperatures. It is tempting to consider our data in view of a recent study in which the light response of native and artificial pigments was monitored by following the vertical (*Z*) deflection of the tip of an atomic force microscope (Rousso et al., 1997). Interestingly, pigments II–IV exhibited a positive atomic force signal on a 10^{-4} to 10^{-3} s time scale, analogous to that observed in the case of native bR. The effect was interpreted in terms of a light-induced structural change in the protein that is not accompanied an optical photocycle, namely, by changes in the optical spectrum of the retinyl chromophore. The membrane-protein conformational alterations were monitored on a 10^{-4} to 10^{-3} s time scale, but were probably triggered by much faster events initiated in the retinal binding site. Such conformational alterations may be responsible for the light-induced reaction with hydroxylamine. Although there is no direct evidence to independently confirm this hypothesis, it is tempting to suggest that after coupling to the photocycle events (Rousso et al., 1997), these structural changes may play a role in catalyzing the hydroxylamine reaction in the case of native bR as well. Future studies should address this possibility. The analogy with observations in the case of a locked rhodopsin (Bhattacharya et al., 1992b) may indicate that similar structural changes are also induced by light in visual pigments. We note that protein conformational alterations induced by retinal light absorption was also suggested in the process of retinal binding to apoprotein at low humidity (Rousso et al., 1996). In this process, critical cavities in the protein are temporarily open, despite the fact that the retinal-protein complex lacks a characteristic photocycle.

We finally consider the mechanism of the hydroxylamine reaction in the dark and its enhancement by light. The striking analogy between the rate parameters (high E^{l}_{a} and high A^{l}) of pigments I and IV (see Table 1) suggests an analogous mechanism that differs from those (low E^{l}_{a} and low A^{l}) of pigments II and III. This may be in keeping with the distorted protein conformation at the binding site of the latter pigments (Rousso et al., 1995), as compared to the relaxed conformation associated with the structurally similar chromophores in I and IV. It is plausible that in native bR, which in the dark is composed of 40%/60% 13-*cis*/all-*trans* isomers (Scherrer et al., 1989), the reaction proceeds via the 13-*cis* component, which is geometrically similar to the chromphore in IV.

A different situation prevails in the case of the light-catalyzed reaction, for which we observe an increase in E^{l}_{a} as compared to E^{d}_{a} for all locked (II–IV) molecules. This is

in clear contrast to the native pigment, where $E^{l}_{a} \ll E^{d}_{a}$. It appears that coupling to the photocycle and/or the specific native protein conformation which binds all-*trans* retinal, compensates for the low E^{l}_{a} value by a much lower A^{l}, keeping the light-catalyzed process at a relatively low efficiency. In other words, the conformational alterations induced by light increase the probability that the reaction will occur. However, coupling to the photocycle or the specific protein conformation compensates for this effect. Future work should aim at establishing the light-induced reaction mechanism, primarily clarifying the nature of the primary step (e.g., reaction of the Schiff base with a water molecule, as compared with a direct, single-step, reaction with hydroxylamine), and correlating the relevant structural changes with those responsible for the photobiological function of bR. Finally, it will also be of interest to clarify the reactions between such mechanistic issues in bR and those of the analogous reactions in visual pigments.

This work was supported by grants from Rakia I Hatch, the Fund for Basic Research (administered by the Israel Academy of Sciences and Humanities) Centers of Excellence Program, from the U.S.–Israel Binational Science Foundation, and from the Israeli Ministry of Sciences and Arts through its strategic infrastructure grants program.

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