Bacteriorhodopsin Experiences Light-induced Conformational Alterations in Nonisomerizable C_{13}=C_{14} Pigments
A STUDY WITH EPR

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The mechanism by which bacteriorhodopsin is activated following light absorption is not completely clear. We have detected protein conformational alterations following light absorption by retinal-based chromophores in the bacteriorhodopsin binding site by monitoring the rate of reduction-oxidation reactions of covalently attached spin labels, using EPR spectroscopy. It was found that the reduction reaction with hydroxylamine is light-catalyzed in the A103C-labeled pigment but not in E74C or M163C. The reaction is light-catalyzed even when isomerization of the C_{13}=C_{14} bond of the retinal chromophore is prevented. The reverse oxidation reaction with molecular oxygen is effective only in apomembrane derived from the mutant A103C. This reaction is light-accelerated following light absorption of the retinal oxime, which occupies the binding site. The light-induced acceleration is evident also in “locked” bacteriorhodopsin in which isomerization around the C_{13}=C_{14} bond is prevented. It is evident that the chromophore-protein covalent bond is not a prerequisite for protein response. In contrast to the case of the retinal oxime, a reduced C=N bond A103C-labeled pigment did not exhibit acceleration of the oxidation reaction following light absorption. Acceleration was observed, however, following substitution of the polypeptide by groups that modify the excited state charge delocalization. It is suggested that protein conformational alterations are induced by charge redistribution along the retinal polyene following light absorption.

Bacteriorhodopsin (bR) is the integral protein of the purple membrane of *Halobacterium salinarum* and serves as a light-driven proton pump (1–3). It is composed of seven transmembrane helices enclosing the binding pocket for an all-trans-retinal chromophore, which is bound to Lys216 via a protonated Schiff base (SBH)†. Absorption of a photon by the retinal induces an all-trans → 13-cis isomerization, which initiates a photocycle with several distinct spectroscopic intermediates, J_{625}, K_{590}, L_{550}, M_{412}, N_{560}, and O_{640}. It is well established that the retinal in K_{590} is characterized by a 13-cis configuration (4,5). Deprotonation of the protonated Schiff base takes place during the L to M transition, which is accompanied by protonation of Asp^{56} and the appearance of a proton at the extracellular surface. The Schiff base is reprotonated during the M to N transition from the proton donor Asp^{96}, which is finally reprotonated from the cytoplasmic side during the recovery initial state of bR.

It is widely assumed that all light-induced protein conformational alterations in retinylidine proteins, including bR, are initiated by isomerization of the retinal chromophore. However, alternative approaches have been suggested in which isomerization is not the only trigger for biological activity or protein structural alterations (6). One approach attributed protein conformational alterations to large charge redistribution in the retinal chromophore developed following light absorption (7–9). In keeping with these suggestions, we have recently shown, using atomic force sensing (AFS), that protein conformational alterations are induced in bR following light absorption, even when the crucial C_{13}=C_{14} double bond isomerization is prevented by a rigid ring structure (10). Thus, the data bring into question (providing direct experimental results) the hypothesis that all primary events in retinal proteins are due to an initial *trans* → *cis* isomerization. Furthermore, we have recently examined the light-catalyzed cleavage of the retinal-protein covalent bond by hydroxylamine (HA) (11) and concluded that the reaction was caused by light-induced conformational alterations, extending to a μs to ms time scale, which are not due to an optically detectable photocycle, which is associated with C_{13}=C_{14} isomerization.

In this work, we apply a new and independent approach, the rate of reduction-oxidation of covalently attached spin labels, to detect protein conformational changes induced by light absorption by retinal-based chromophores in the intact binding site. We have followed the site-directed spin labeling method (12, 13), which offers an approach to identify structural changes in bR at different parts of the protein with millisecond time resolution (14–18). This method was also used to determine the identity and orientation of the secondary structure of protein domains and the topography of polypeptide chains with respect to the membrane solution interface (19, 20). The topography of bR was mapped using site-directed spin labeling by determining the collision rate of the nitroxide side chain with a freely diffusing paramagnetic reagent, one polar and the other nonpolar. The polar reagent is usually a chromium oxalate localized in the aqueous phase, and the nonpolar is molecular oxygen localized in the lipid phase (21).

In the present study, we used site-directed spin labeling to probe light-induced conformational changes in bR and in artificial locked pigments (Scheme 1), which were labeled with an EPR probe at positions 74 (E74C mutant) at the extracellular
side and A103C and M163C at the cytoplasmic side (22–28). We took advantage of the possibility to reduce the nitroxyl radical with HA (Scheme 2), which is followed by the disappearance of the EPR signal, and of the subsequent spontaneous oxidation of the reduced radical with molecular oxygen.

The experimental results show that, in the case of the A103C-labeled mutant, the reduction of nitroxyl radical with HA is light-sensitive even when the C13=C14 bond is locked to isomerization. We have also studied the intriguing question as to the necessity of the retinal-opsin covalent bond for protein isomerization. It has been shown previously that a pigment devoid of a retinal-opsin C=N bond is formed upon reconstitution of K216A (29) and K216G (30) mutants with all-trans-retinal Schiff bases. Moreover, this noncovalent bound chromophore exhibits a photocycle analogous to that of native bR. In the present study, we have detected protein response to light absorption of retinal oxime, which occupies the binding site but does not covalently bind to the protein. The response was also detected in “locked” chromophores in which isomerization of the retinal-opsin covalent bond takes place. In variance with the retinal oxime system, a reduced Schiff base chromophore does not exhibit a light-induced oxidation reaction of the spin label. However, such a reaction is observed upon asymmetrically substituting the polyene with groups that modify the excited state charge delocalization.

MATERIALS AND METHODS

Sample Preparation—The spin label (1-oxyl-2,2,5,5-tetramethylpyrrol-3-ylmethanethiosulfonate (MTSSL) in dimethyl sulfoxide; Toronto Research Chemicals, Canada) was covalently attached to the cysteine residue of the appropriate bR mutant (A103C, M163C, or E74C) to yield the spin label side chain. A 10-μl solution of 100 mM MTSSL in dimethyl sulfoxide was diluted with a 2-ml suspension of 50 μM bR mutant 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. The EPR spectra of the spin-labeled bR was measured to estimate the extent of labeling and to assure removal of nonbound spin label.

Preparation of Spin-labeled Artificial Pigments—Retinal analogs were prepared as described previously (32, 33). The 103C apomembrane was prepared from the A103C mutant pigment according to an established method (34). This apomembrane was incubated with 1.2 equivalents of the retinal analogs (Scheme 1) at pH 7 for 3 days with retinal analog 2 and 12 h for analogs 3 and 4. The artificial pigments derived from A103C mutant were spin-labeled as described above to yield the 103 spin label side chain.

Reduction of the Spin-labeled Pigments with Hydroxylamine—The spin label in bR mutants (A103C, M163C, and E74C) or in the appropriate artificial pigment (Scheme 1, II, derived from analog 2) was reduced by the addition of 0.2 μM HA at pH 7 to a 70 μM solution of the pigment suspension (Scheme 2). The reduction reaction was monitored by the disappearance of the central component of the EPR spectrum. 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 510-nm glass cut-off filter. Illumination was kept steady for the whole experiment.

Oxidation of Spin-labeled Apomembrane—Spin-labeled bR and the artificial pigments (Scheme 1, II, III, and IV, derived from analogs 2–4) were bleached to produce the apomembrane by mixing 1 μM HA at pH 7 with 15 μM pigment suspension. The suspension was illuminated with a 150-watt halogen lamp using a 510-nm cut-off filter and was monitored by the disappearance of the main absorption band (570 nm for II and III and 550 nm for IV). The spin label was completely reduced during the reaction (Scheme 2), faster than the pigment bleaching process as described above. The HA was removed by washing the pellet four times with water. The oxidation reaction by molecular oxygen took place spontaneously following HA removal (Scheme 3), and the reaction was monitored by following the increase in the central component of the EPR spectra. Experiments were carried out in the dark and under blue light illumination with a window filter (360 nm < λmax < 420 nm). The initial EPR signal intensity prior to illumination was relatively high due to spontaneous oxidation of the spin label in the dark, which took place during the sample preparation HA treatment (~2 h).

Oxidation of Spin-labeled Reduced Pigments—A103C bR mutant was bleached to produce the apomembrane. Artificial pigments were prepared by incubating 1.2 equivalents of the retinal analogs (Scheme 1) with A103C apomembrane at 25 °C pH 7. The Schiff-base bond of wild type bR and of the artificial pigments was reduced by Sodium borohydride to produce the polyene chain covalently bound to the protein. The reaction was carried out under illumination with a 510-nm cut-off filter and was monitored by the disappearance of the main absorption peak of each pigment. The sodium borohydride was removed by washing the membrane pellet with water four times. The reduced pigments were spin-labeled as described above, and the membrane pellet was washed four times with a solution of 1% bovine serum albumin. The spin-labeled reduced pigments were incubated for 30 min with 0.5 μM HA in the dark to reduce the spin label side chain. The HA was removed by washing the membrane pellet four times with water followed by EPR measurements to monitor the spontaneous oxidation of the reduced spin label. The reaction was monitored by following the increase in the central component of the EPR spectra in the dark and under illumination using white light with a 310-nm cut-off filter.

EPR Measurements—All measurements were performed on a Bruker D-SRC, ER 290 spectrometer, using a flat cell (volume 60 μl) at 23 °C. Conditions were as follows: center of field, 3500 gauss; modulation amplitude, 1.0 gauss; microwave power, 20 milliwatts; receiver gain, 3 × 105 to 8 × 105. The kinetics were measured by monitoring the central component at a fixed position.

RESULTS

Reduction Reaction of Spin-labeled Pigments—It was previously shown (35) that nitroxide spin labels can be easily reduced by hydroxylamine (HA) and are reoxidized by molecular oxygen following exposure to air. Bacteriorhodopsin was labeled with a nitroxy radical at positions 103 and 164 in the cytoplasmic side and 74 in the extracellular surface of the protein by reaction of the appropriate mutants with MTSSL in dimethyl sulfoxide. HA was added to the protein, and the reduction of the radical was monitored by a decrease in the peak intensity of the central component of the EPR spectra. The rotational correlation times for the spin labels of 74, 103, and 164 were 8 × 10−10 s, 8 × 10−8 s, and 3 × 10−9 s, respectively, as calculated according to the method described in Ref. 36.

The reaction rate was followed in the dark and under illumination with light, which is absorbed exclusively by the retinal chromophore. The reaction was carried out in the presence of a relatively low concentration of HA (0.2 μM), which was sufficient to reduce the radical without significant bleaching of the pigment (5% bleaching was measured following illumination with a 510-nm cut-off filter). We found that, for labeled bR
at position 103 in the cytoplasmic side, the reaction was accelerated by light ~1.5 times, relative to the dark under the conditions used in the experiments (Fig. 1).

The reaction in the A103C-labeled system was further carried out in an artificial "locked" pigment derived from chromophore 2 (Scheme 1), in which isomerization around the C13=C14 double bond is prevented. It was found that the reaction rate was accelerated by light by a factor of 1.4, similar to the extent of acceleration detected in the labeled native bR. In contrast to labeling at Cys103, labeled mutants E74C and M163C did not show any observable light acceleration of the reduction rate. Removal of the hydroxylamine reagent did not induce spontaneous oxidation of the spin label at any of the three labeled pigments in the dark, nor following illumination.

**Scheme 3**

Oxidation of the Reduced Spin Label in Apomembranes—

The addition of a high concentration (1 M) of HA to the A103C-labeled pigment induced two reactions. The first involves reduction of the radical (as described above), whereas the second is associated with cleavage of the protonated Schiff base linkage to produce an apomembrane in which a retinal oxime molecule replaces the covalently bound retinal in the binding site (11). Following removal of the HA reagent from the apomembrane, a spontaneous oxidation of the reduced radical by oxygen occurs following exposure to air, according to Scheme 3.

The oxidation reaction in native bR was monitored by an increase in the EPR signal. Its significant acceleration by illumination with blue light (360 nm < \( \lambda_{\text{max}} < 420 \text{ nm} \)), which is absorbed by the retinal oxime was clearly observed (Fig. 2A). In the apomembrane derived from the A103C mutant, the reaction could be accelerated as much as by a factor of 4.5 times. In contrast, we did not observe light acceleration in apomembranes derived from M163C- and E74C-labeled mutants. We note that the line width of the EPR signal did not change during the experiment, and only the peak intensity increased. This indicates that the reappearance of the EPR signal is associated with a chemical reaction that produces the nitroxyl radical (Scheme 3) and not with environmental alterations. To support the assumption that the reaction acceleration is associated with light absorption by retinal oxime (\( \lambda_{\text{max}} = 360 \text{ nm} \)), we irradiated the sample with a 460-nm cut-off filter but did not detect the reaction acceleration, in keeping with the assumption that the acceleration is associated with light absorption by the retinal oxime chromophore.

The oxidation reaction was also investigated with A103C-labeled apomembranes prepared from artificial labeled pigments derived from analogs 2, 3, and 4 (Scheme 1, Fig. 3). All three artificial pigments are characterized by a C13=C14 double bond "locked" by a rigid ring structure. Although isomerization of this "critical" bond is now precluded, the label oxidation rates of all three "apo-locked" pigments were found to be accelerated by light absorption of the retinal oxime. The effects were comparable with those of the native pigments. As summarized in Table 1, under the same illumination conditions, apo-locked cis (III) was accelerated 6 times, apo-locked trans (II) 5.5 times, and apo-locked furan (IV) 4.4 times.

An interesting effect was observed in the dark reaction that followed irradiation. The oxidation reaction rate still accelerated although illumination was stopped, and it gradually diminished in ~5 min, returning to the original rate, as measured under dark conditions. This "postillumination effect" (Fig. 2B) was observed in all of the labeled apomembrane samples that were studied.

**Oxidation of the Reduced Spin Label in Reduced C=N Bond Pigments**—To obtain further insight into the origin of the light-catalyzed oxidation and to evaluate the possibility that light-induced conformational alterations in the protein are associated with light-induced retinal charge redistribution, we carried out experiments in systems in which the light-induced dipole in the polyene is substantially modified. In view of the critical role played by the protonated Schiff base in generating the AFS signals attributed to the light-induced dipole (10), we worked with systems in which the C=N bond was reduced with sodium borohydride, followed by labeling of the appropriate cysteine residue with a nitroxyl radical. In the reduced system, the retinal polyene is practically symmetrical, and, as suggested by the lack of light-induced Atomic Force Sensing (AFS) signal (10) and by Second Harmonic Generation (SHG) studies, \( 2 \) it is devoid of a significantly induced dipole. The C=N reduced labeled protein was subjected to HA (0.1 M) treatment, which reduced the radical. Oxidation of the reduced radical (following HA removal) was monitored in the dark and under illumination.

In contrast to the case of retinal oxime, exposure of the reduced retinal chromophore of the A103C-labeled pigment to white light did not induce any acceleration of the oxidation reaction (Fig. 4A). The effect of light on the oxidation rate of the reduced radical was further examined in three groups of labeled and reduced artificial pigments.

The first group included reduced pigments substituted by electron-donating or -withdrawing groups located in the vicinities of the C5 atom (Scheme 4). The oxidation reaction of the 14-F analog was accelerated 6-fold by light, and the 13-CF3 was 5 times faster (Fig. 4B).

The second group of reduced pigments is characterized by substituents located at the opposite end of the polyene chain. The artificially reduced pigments bearing 5-dimethylamino-benzene (X) and N-amine (XI) polyenes both showed light acceleration of the radical oxidation (Fig. 4B).

We interpret the above observations in terms of substituent-induced restoration of the excited state dipole, which is absent in the symmetrical, reduced chromophore of the native retinal. To further examine this conclusion, we investigated a third group of C=N reduced pigments, substituted by electron-donating groups (VII–IX) at the center of the polyene chain. Analogously to the reduced native pigment, none of these species exhibited any light effect on the oxidation reaction (Fig. 4A).

To exclude the possibility that the light acceleration oxidation reactions with reduced pigments originated from the residual retinal oxime produced during the bR bleaching reaction, we performed a control experiment with bleached bR reduced with sodium borohydride, which was labeled with the EPR probe. No light acceleration was observed. Since in these systems the retinal binding site is occupied by the covalently bound reduced polyene, this observation is in keeping with the interpretation that a light-induced effect is observed only upon excitation of oxime molecules, which are located in the retinal binding site.

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\(^2\) A. Lewis, A. Khachatryan, I. Rousso, and M. Sheves, unpublished results.
DISCUSSION

Chemical reactions in bR and their possible light acceleration are important tools for detecting structural changes in the protein following light absorption (11). While previous studies approached this issue via the HA light-induced reactions of the Schiff base, the present work detects light-induced conformational alterations in protein domains other than the retinal binding site. This was carried out by applying the spin label redox methodology in order to explore the occurrence of light-induced protein conformational alterations, which are not initiated by isomerization of the C13−C14 double bond.

Site-directed spin labeling was widely used to obtain information of the label environment and alterations in protein conformation during the photocycle (12–19). Here we have used chemical reduction reactions of the nitroxyl radical with HA and subsequent oxidation with molecular oxygen to reform the radicals as tools for probing light-induced protein conformational alterations in a variety of artificial and chemically modified chromophore systems.

Among the three bR mutants studied (A103C and M163C on the cytoplasmic side and E74C on the extracellular side), only the A103C exhibited significant acceleration of the reduction reaction upon light absorption by the retinal chromophore. This effect might be related to previous observations, showing that the protein experiences conformational alterations in the vicinity of the 103 residue that are not essentially associated with the photocycle and therefore do not require C13−C14 double bond isomerization. A different situation prevails in the vicinity of residues 163 and 74. Apparently, the protein does not experience substantial conformational alterations around these residues following light absorption, or alternatively, such changes do occur but do not enhance the reduction reaction.

We note that in contrast to A103C and M163C-labeled pigments (16), the EPR line shape of the radical in the E74C mutant located in the extracellular surface in an interhelical loop (22–28) exhibits a narrow line width and a single, hyperfine splitting, implying relatively high mobility of the radical. However, the chemical reaction to form the label pigment was very slow, indicating less accessibility of the reagent. It is important to note that unlike the reduction reaction with hydroxylamine, the back-oxidation does not occur under both dark and light conditions. This is in keeping with previous work (21) that indicates that position 103 and 163 mutants are not accessible to collisions with molecular oxygen, since they are located at the border between the protein and aqueous phase. Our present work indicates that oxygen accessibility is not sufficiently increased in the vicinity of these residues under light conditions. The different reactivity of the spin label probes toward hydroxylamine and oxygen can be rationalized by their different polarities, which affect their accessibility. Thus, the polar hydroxylamine reacts with the spin label probes in contrast to the apolar oxygen, reflecting a polar environment of the probes.

A significant difference is observed when the oxidation reaction is investigated in the apoprotein, where the original retinal Schiff base is replaced by a retinal oxime. In this case, slow oxidation with molecular oxygen is observed in the dark for the vicinity of the 103 residue that are not essentially associated with the photocycle and therefore do not require C13−C14 double bond isomerization. A different situation prevails in the vicinity of residues 163 and 74. Apparently, the protein does not experience substantial conformational alterations around these residues following light absorption, or alternatively, such changes do occur but do not enhance the reduction reaction.

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label at the 103 residue, which is considerably accelerated following irradiation of the retinal oxime, absorbing at 360 nm. It was previously shown that retinal oxime occupies the retinal binding site and exhibits a CD signal (38). Moreover, AFS studies detected protein conformational alterations induced by light absorption by the retinal oxime. Thus, the light-accelerated oxidation of the spin label provides further evidence that light absorption by the retinal oxime induces a protein conformational alteration, in this case an alteration that increases oxygen accessibility to the 103 region, thus allowing the oxidation reaction. It is evident that the protein conformation of the apoprotein is different from that of native bR, at least around the 103 residue, and that such differences are responsible for different oxygen accessibility to the vicinity of this residue. Furthermore, these light-induced protein conformational alterations do not require a chromophore-protein covalent bond; i.e. chromophore occupation of the binding site is sufficient for generating light-induced structural changes in the protein. This feature is reminiscent of the behavior of bacteriorhodopsin pigments lacking the retinal–Lys216 covalent bond (29, 30) that were prepared by reconstituting K216G and K216A mutants with retinal alkylamine Schiff bases. The pigments exhibited the basic photochemical features of native bR as well as the associated proton pumping activity.

The light-induced conformational alterations prevailing in the native apomembrane that contains retinal oxime as its chromophore are also present in artificial apomembranes derived from retinal analogs 2–4. In these chromophores, the isomerization around the C13=C14 double bond (in 2 and 3) and around C13=C14 and C11=C12 in 4 is precluded. Thus, it is evident that such protein structural changes are not associated with isomerization of these two double bonds. Although isomerization around the C9=C10 bond cannot be definitely excluded, it seems highly unlikely in view of the comparable catalytic light effect in the case of the two trans locked chromophores (2 and 4) and of the cis locked chromophore (3). Since a double cis photoproduct is highly unlikely in retinal Schiff bases (39), C9=C10 isomerization will not be in keeping with the comparable effects of trans and cis systems.

An interesting effect is associated with the “postlight effect” (Fig. 2B). The oxidation reaction kinetic reaches its dark value after only a few minutes following cessation of light illumination. We suggest that the oxygen remains “trapped” in the

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3 I. Rousse, E. Khachatryan, A. Lewis, and M. Sheves, unpublished results.
vicinity of the 103 residue for a relatively long time after the protein has retained its original conformation, due to existing barriers for the diffusion of the oxygen outside. It is concluded that a channel for oxygen connecting the 103 residue vicinity with the outside medium exists also in the dark, but light raises the affinity of the 103 residue protein vicinity to oxygen and possibly decreases the barrier for oxygen penetration. The absence of the effect of light of the oxidation reaction in mutants M163C and E74C might be explained by a lack of protein changes in these domains or by low accessibility of oxygen, even under light illumination.

Reduction of the protonated Schiff base protein linkage with sodium borohydride leads to a symmetric polyene covalently bound to the protein in which significant electronic charge redistribution following light absorption can be excluded. This pigment did not show any light-catalyzed oxidation reaction. This observation is consistent with the mechanism suggested for the retinal oxime light catalysis, namely that the catalytic conformational alterations are associated with charge redistribution following light absorption. Compelling support for charge redistribution as the cause of conformational alterations is gained by experiments with substituted reduced polyenes. Substitution with withdrawing electrons (F, CF$_3$), close to the Schiff base linkage, induced light oxidation acceleration; similarly, introducing electron-donating groups instead of the $\beta$-ionone ring initiated protein response as well. This indicates that the asymmetric electronic distribution causes a catalytic effect analogous to the oxime systems. Interestingly, no protein response could be detected in polyenes in which withdrawing or donating groups were located in the middle of the polyene chain. Finally, we note that the lack of light-catalyzed reaction in the symmetric chromophore and its presence in the substi-

### Table 1

| System | Light acceleration |
|--------|--------------------|
|        | Reduction | Oxidation |
| 103C$^a$ I | 1.5 | —$^b$ |
| 103C$^a$ II | 1.4 | — |
| 163$^a$ I | 0 | — |
| 74$^a$ I | 0 | — |
| 103C$^c$ I | 4.5 | |
| 103C$^c$ II | 5.5 | |
| 103C$^c$ III | 6.0 | |
| 103C$^c$ IV | 4.4 | |
| 163$^c$ I | 0 | |
| 74$^c$ I | 0 | |
| 103C$^d$ I | 0 | |
| 103C$^d$ V | 6 | |
| 103C$^d$ VI | 5 | |
| 103C$^d$ VII | 0 | |
| 103C$^d$ VIII | 0 | |
| 103C$^d$ IX | 0 | |
| 103C$^d$ X | 3.4 | |
| 103C$^d$ XI | 0 | |

$^a$ 0.2 M HA, $\lambda > 510$ nm.
$^b$ Oxidation reaction undetectable.
$^c$ Oxidation reaction in apomembrane 360 nm < $\lambda < 420$ nm. Chromophore oxime is irradiated.
$^d$ Radical formation by oxidation reaction in reduced C=N pigments, cut-off filter. $\lambda > 310$ nm.

FIG. 3. A, EPR spectra of reoxidation by molecular oxygen of A103C spin-labeled apomembrane derived from pigment III (locked 13-cis, 3). Spectra were taken at intervals of 5 min. Spectra 1 and 2 were monitored in the dark, and spectra 3–5 were monitored under illumination. B, progress of the reoxidation reaction under light and dark conditions, measured by EPR signal intensity of A103C spin-labeled apomembrane of C$_{13}$–C$_{14}$-locked bR pigments II–IV. The signal intensity was monitored at the central component of the EPR spectra.

**TABLE 1**

*Acceleration by light of the spin label reduction (by hydroxylamine) and oxidation (by O$_2$).*

Values are relative to a nonilluminated solution under identical conditions.

**FIG. 4.** Kinetics of reoxidation under light and dark conditions, monitored at the central component of the EPR spectra. A, reduced C=N bond of A103C-labeled pigments derived from native retinal I and pigments VII–IX. B, pigments V and VI and pigments X and XI.
tuted, asymmetric systems excludes the possibility that protein structural changes are induced by excess light energy dissipated as heat.

It is tempting to relate our present results to a previously described study in which light response of bR and locked artificial pigments was monitored by AFS (10). The effect detected in the locked pigments was interpreted in terms of a light-induced structural change in the protein that is not accompanied by an optical photocycle. Analogous changes may play a role in catalyzing the light-induced HA reaction (11) of the Schiff base as well as the reactions of the probe at the Cys103 position described above. Protein conformational alterations induced by light absorption were also suggested in the process initiated by 9-cis-retinal isomerization to all-trans of retinal binding to apoprotein at low humidity, which was induced by light absorption were also suggested in the process described above. Protein conformational alterations and those detected during the bacteriorhodopsin photocycle. Analogous changes may play a role in catalyzing the light-induced HA reaction (11) of the Schiff base as well as the reactions of the probe at the Cys103 position described above. Protein conformational alterations induced by light absorption were also suggested in the process initiated by 9-cis-retinal isomerization to all-trans following light absorption (40). Critical cavities in the protein are opened following light absorption by all-trans-retinal (following the isomerization of 9-cis to all-trans), despite the fact that the retinal-protein complex lacks a characteristic photocycle.

In conclusion, we have shown that the protein experiences conformational alterations following light absorption that are not associated with double bond isomerization but are instead due to charge redistribution developed in the retinal chromophore. A chromophore-protein covalent bond is not a prerequisite for the protein response. A future study should clarify whether a relationship exists between these conformational alterations and those detected during the bacteriorhodopsin photocycle as well as clarifying their possible importance in the bacteriorhodopsin function.

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