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Spin-labeling analysis of structure and dynamics in octopus rhodopsin

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Abstract

The location of cysteines accessible in octopus rhodopsin were characterized by a spin-labeling technique. Two cysteines were found to bind a methanthiosulfonate spin label. One of the spin labels is attached to helix V with the side chain located within the membrane, most probably close to the polar head group region. The second spin label was found to be attached to cysteine 345 in the C terminus. Light-induced reversible electron paramagnetic resonance spectral changes were observed for the spin label attached at position 345. It is concluded that conformational changes occur during the rhodopsin to metarhodopsin transition in the vicinity of the C-terminus position 345.

Keywords: Rhodopsin; Electron spin resonance spectroscopy; Spin labeling

1. Introduction

Rhodopsin, the major integral membrane protein of vertebrate and invertebrate photoreceptors, is certainly one of the best-studied receptor molecules. It consists of a single polypeptide chain which traverses the membrane in seven α helices. Approximately in the center of the protein opsin, the chromophore 11-*cis* retinal is located in a hydrophobic pocket, where it is covalently linked to a lysine residue via a protonated Schiff-base linkage.

Absorption of light leads to the *cis*-to-*trans* isomerization of the chromophore which triggers a sequence of fast conformational changes with discrete, optically well-characterized intermediates. One of the intermediates (vertebrates, metarhodopsin II; invertebrates, acid metarhodopsin) elicits a biochemical cascade which eventually leads to an amplified electrical signal of the photoreceptor cell [1]. This signal is then transmitted to higher order neurons.

The photochemical events in invertebrate rhodopsin are similar to those of vertebrate rhodopsin, except that light does not bleach rhodopsin but converts it to a rather thermostable photoproduct metarhodopsin which can be reconverted by light to the parent pigment (photoregeneration). This metarhodopsin may exist in two forms, acid ($\lambda_{max}=514$ nm) and alkaline metarhodopsin ($\lambda_{max}=380$ nm) depending on the pH of the sample [2,3].

In order to understand better the functioning of a receptor molecule at the molecular or even atomic level, the conformational changes which finally lead to the exposure of a site which allows the signal molecule to bind have been the subject of numerous studies. Various technique such as linear and circular dichroism, biochemical methods, Fourier transform IR (FTIR) spectroscopy especially in combination with site-directed mutagenesis have been used to elucidate the structure–function relationship. Recently, electron paramagnetic resonance (EPR) spectroscopy became a powerful tool, especially since the former requirement of the presence of natural cysteine(s) in the protein is no longer essential; mutagenesis now allows one to introduce cysteines in any selected region of a protein to which cysteine-specific spin labels are covalently bound. This technique has already been successfully applied to vertebrate rhodopsin and bacteriorhodopsin and has led to new and exciting information on the structure and dynamics of these two retinal-binding proteins [4,5]. Moreover, the information obtained from experiments in which cysteines were carefully and systematically introduced into a protein may be used to interpret data which come from natural cysteines, as will be shown later.

Here, we present preliminary EPR data obtained from spin-labeled octopus rhodopsin. According to the amino acid sequence, octopus rhodopsin has eight cysteines [6]. Our major aim in the present paper is to localize those cysteines which are accessible to a methanethiosulfonate spin label, and to obtain structural information regarding the local environment in the vicinity of the spin label binding sites in both the rhodopsin and the acid metarhodopsin conformation.

2. Materials and methods

Eyes from freshly killed *Octopus vulgaris* were enucleated, rapidly frozen and stored at 190 K until use. Frozen eyes were

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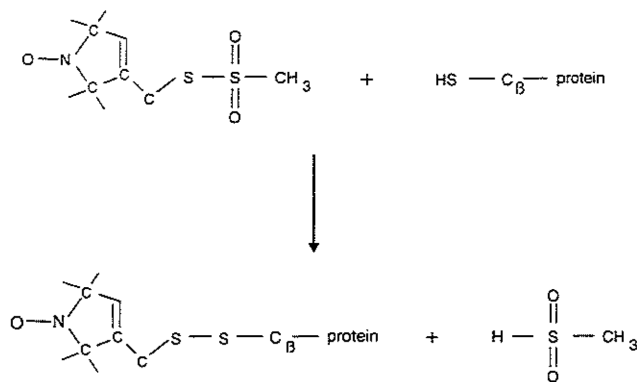

Fig. 1. The spin label MTSSL was used to modify the accessible cysteines of octopus rhodopsin to yield a spin-labeled side chain.

then carefully thawed, the retinae removed, and the photoreceptor membranes isolated according to the method described by Kito et al. [7]. All procedures were carried out at 4 °C and in dim red light. In order to make sure that the membranes contain only rhodopsin, the resulting membrane fraction was additionally adapted to light of 600 nm wavelength. Spectrophotometry of unlabeled and labeled photoreceptor membranes was carried out with a Hitachi 356 spectrophotometer. The samples were irradiated with light from a xenon arc (150 W) passed through an interference filter (Schott & Gen., Mainz, Germany). Flash irradiation was performed by means of an eximer pumped dye laser (Lambda Physik, Göttingen).

The spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTSSL) (Fig. 1) was purchased from Reanal (Budapest). For spin labeling of the membrane-bound rhodopsin, 100 mM MTSSL in acetonitrile was injected into a 0.5 ml suspension of membrane in 100 mM phosphate buffer (ph 7.4), leading to a label:rhodopsin ratio of 10:1. The samples were incubated at room temperature for 12 h. The non-covalently bound spin label was removed by centrifugal washing (four times at 10 000 g for 10 min) with distilled water, followed by four washings with 100 mM phosphate buffer (pH 5.4). All procedures were carried out in dim red light. The EPR spectra were recorded with a home-made X-band EPR spectrometer equipped with a Bruker dielectric resonator and a Bruker B-NM 12 *B*-field meter. Samples were loaded in quartz capillaries. After analog to 12 bit digital conversion the spectra were processed in a personal computer.

3. Results and discussion

3.1. Spin labeling of octopus rhodopsin

EPR spectroscopy of spin-labeled octopus rhodopsin reveals a complex spectrum with the low and high field line shapes of multicomponent properties (Fig. 2, curve a). The stoichiometry is found to be two spin labels bound per rhodopsin molecule, which agrees with the earlier finding that two sulfhydryl groups are titrated per membrane-bound rhodopsin [8]. Incubation of the membrane suspension with 50 mM of ascorbate reduces that fraction of the spin labels which is accessible to ascorbate ions; half of the attached spin label is reduced within the first few seconds of incubation. The resulting spectrum which is due to the remaining intact spin label shows a very strong immobilization of the spin label side chain (Fig. 2, curve b). In the presence of ascorbate, the lifetime of this remaining fraction of the spin label is of the order of several hours. Subtracting this spectrum from the initial spectrum (Fig. 2, curve a) yields the spectrum of the spin labels which are accessible to ascorbate (Fig. 2, curve c). The line shape reveals intermediate immobilization of the spin label side chain and still shows more than one component in the low field and high field regions. Double integration of both spectra (Fig. 2, curves b and c) gives similar values of spin concentration. We conclude that we are dealing mainly with two sites which are accessible to the specific spin label used. The immobilized spin label (Fig. 2, curve b) will be referred to as attached to site 1, and the more mobile spin label is assumed to be attached to site 2. The multicomponent properties still present in the spectrum of site 2 may result from at least two different conformations of the binding site environment, which are in a thermodynamic equilibrium.

The attachment of the spin labels at sites 1 and 2 does not change the absorption band of rhodopsin or metarhodopsin as demonstrated by the difference spectra shown in Fig. 3.

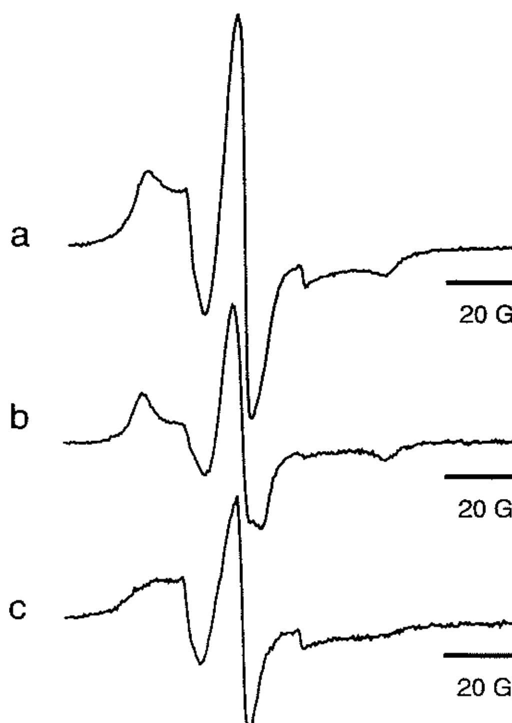

Fig. 2. EPR spectra of spin-labeled octopus rhodopsin at room temperature: curve a, sample with a ratio of spin label:rhodopsin of 2:1; curve b, the same sample in the presence of 50 mM ascorbate; curve c, difference spectrum curve a – curve b.

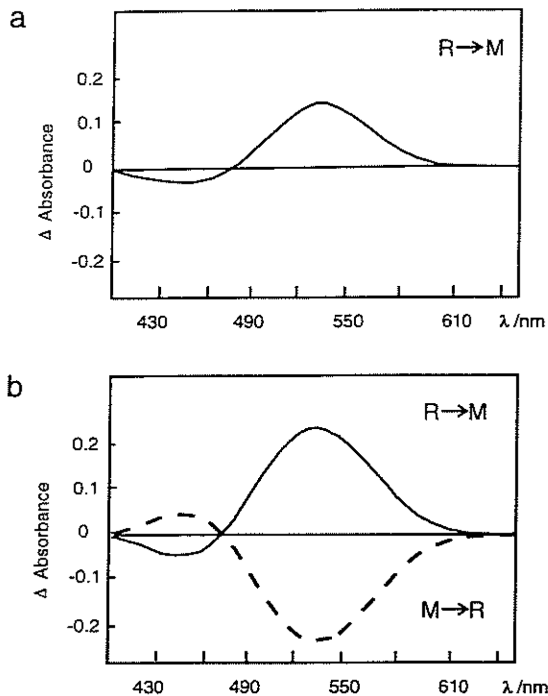

Fig. 3. Optical difference spectra of the interconversion of rhodopsin to metarhodopsin: (a) an unlabeled sample; (b) a spin-labeled sample. The membrane suspension was irradiated at pH 5.4 with light of wavelength 440 nm in (a), and 440 nm (———) and 580 nm (- - -) in (b).

The positions of the maximal absorbance changes and those of the isosbestic points are identical in Fig. 3(a) and 3(b). This indicates that the spin labeling itself does not influence the spectral properties of rhodopsin or metarhodopsin.

3.2. Structural information based on the analysis of the hydrophobicity of the label environment of sites 1 and 2

The hyperfine splitting of spin labels depends significantly on the polarity or hydrophobicity of the spin label environment [9]. This value can be determined from the splitting of the low and high field extremes at temperatures below 200 K. At this temperature the side-chain motion is frozen and the apparent splitting yields twice the value of the hyperfine component A_{zz}. Fig. 4 shows the low temperature spectra of the spin label side chains attached to site 1 and 2. The spectra reveal different hyperfine splittings which are determined by means of a fitting procedure [10]. The value of A_{zz} of site 2 amounts to 35.9 G. This value is found for protein-bound MTSSL with the nitroxide side chain exposed to the aqueous phase. The corresponding value for site 1 is 35.6 G. This decrease in A_{zz} can be explained by a comparably less polar environment of site 1. The comparison of these data with the hyperfine splitting values of MTSSL attached to bacteriorhodopsin D helix mutants shows that the spin label location of site 1 is similar to those attached to positions 105 or 109 of bacteriorhodopsin [11]. The side chains of these amino acids are located within the membrane close to the polar head group region.

3.3. Structural information based on the analysis of the spin label motion

A new promising approach to acquire structural data from the EPR spectrum of spin-labeled proteins is the analysis of the spin label motion. The packing density variation of protein atoms, which depends on tertiary structure in the local nitroxide environments, is reflected in a variation of the mobility of the nitroxide side chain. In areas of high atomic densities such as in the interior of proteins, the spin label motion is spatially restricted owing to multiple interactions with neighboring side chains and backbone atoms. On the outer surface of a helix or loop, however, the spin label motion is relatively unrestricted. Information about the nitroxide mobility is obtained from the EPR line shape at physiological temperatures. The large splitting and the well-resolved high field peak of the spectrum of site 1 reveals a strong immobilization of the spin label side chain (cf. Fig. 2(b)). The spectral shape is similar to that of spin labels located within helix regions where the spin label side chain may strongly interact with the tertiary structure, as for example for spin label position 124 or 127 in bacteriorhodopsin mutants [5].

The side chain of the spin label attached to site 2, however, shows an intermediate immobilization which is found for spin labels located on the outer surface of a helix or within loop regions. The EPR spectrum (cf. Fig. 2(c)) is similar, for example, to the spectra of MTSSL attached to C–D loop positions 105 or 101 of bacteriorhodopsin [5,12].

From the studies discussed above, i.e. the accessibility of the nitroxides to ascorbate, the polarity of the spin label en-

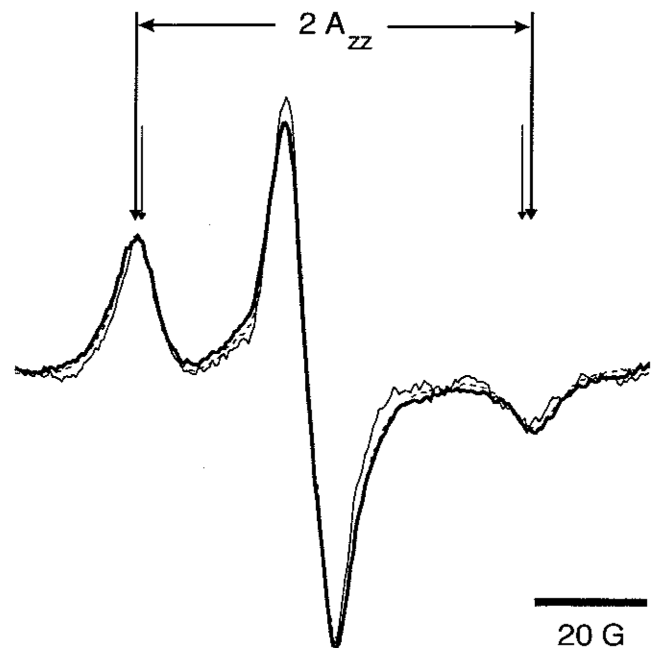

Fig. 4. EPR spectra of spin-labeled octopus rhodopsin at 160 K with the spin label attached to site 1 (———), site 2 (———), and to both sites (- - -). Arrows indicate the high field and low field extremes of sites 1 and 2. The spectrum of MTSSL attached to site 1 shows the smallest hyperfine splitting $2A_{zz}$.

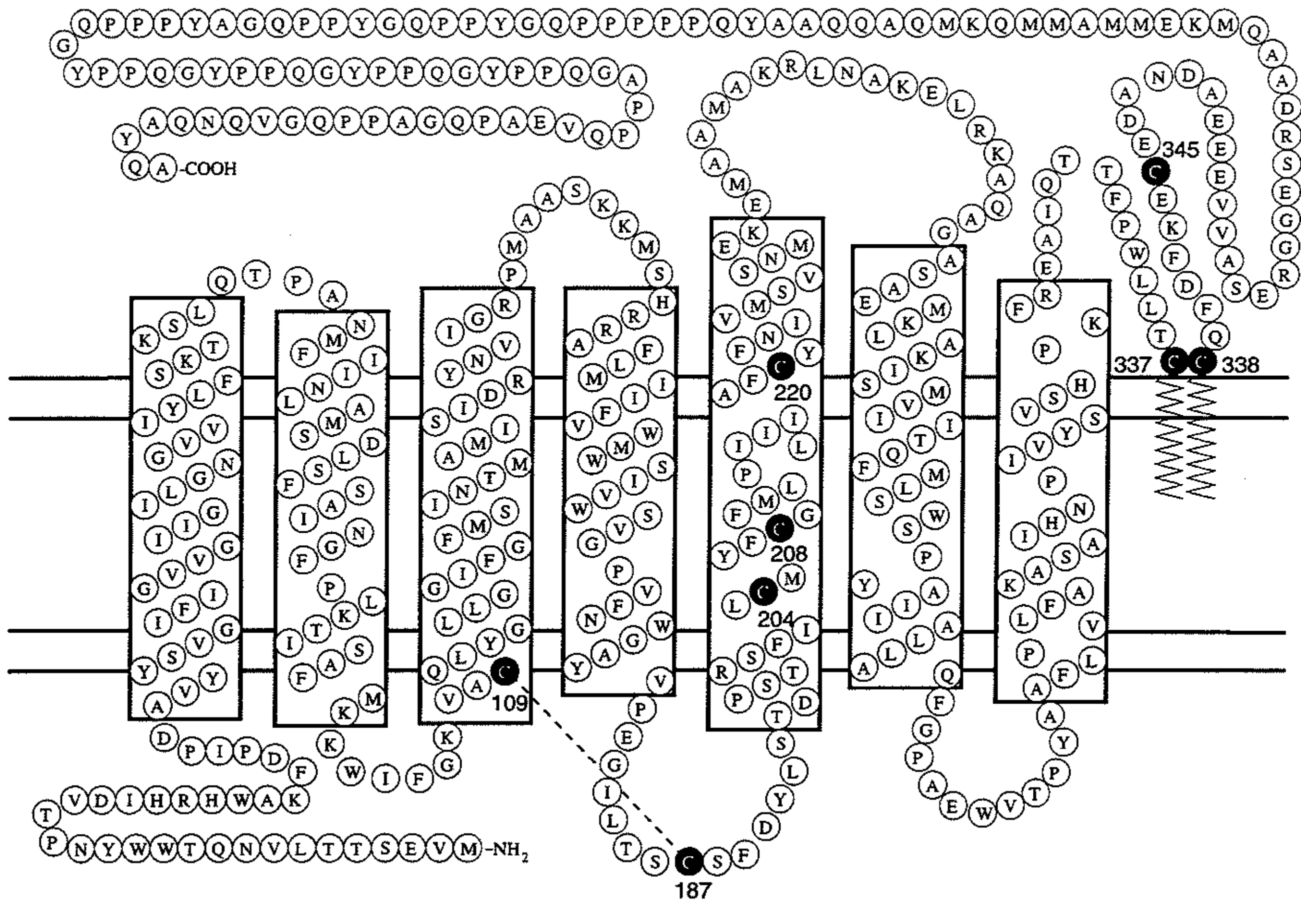

Fig. 5. Model of the transmembrane topography of octopus rhodopsin according to [3,12]: - - -, possible disulfide bond between Cys109 and Cys187. Cysteine residues 337 and 338 are not accessible for spin labels. In accordance with [12], these cysteines are shown as palmitoylated.

vironment and the mobility of the spin label side chains, we conclude that site 1 is located in a helix region within the membrane most probably close to the polar head groups. The respective spectral properties of site 2 are characteristic for the side chain attached to a loop or terminus region located in the aqueous phase.

3.4. Distances between the attached spin labels

The dipolar interaction between attached spin labels leads to a significant line broadening, if the distance between the interacting spins is less than about 2.5 nm. For temperatures below 200 K, the relative motion of the spin label side chains is frozen, and the distance between the spins can be determined from the line width of the spectra. Fig. 4 shows the low temperature spectra for the rhodopsin samples with MTSSL attached to either site 1 or site 2, and to both sites. The linewidth of the EPR spectrum of the spin label attached to site 1 (6.1 G) is significantly smaller than the linewidths of the other two samples. The largest linewidth is found for the EPR spectrum of site 2 (7.6 G), whereas the spectrum of the sample with spin labels attached to both sites is similar to the superposition of the two spectra of site 1 and site 2. For non-interacting spin labels, the linewidth value for protein-bound MTSSL ranges from 5.0 to 6.8 G depending on the local spin label environment. One reasonable explanation for the observed linewidth of 7.6 G found for site 2 is the presence of considerable dipolar interaction of spin labels attached to site 2 of different rhodopsin molecules. If we assume a pair-wise interaction, the distance between these sites is calculated from a spectral fitting [13] to be 2.4 nm. In fact, an occasional appearance of rhodopsin clusters of four to ten molecules in native squid photoreceptor membranes was reported by Saibil and coworkers [14], indicating a semiordered packing of rhodopsin molecules. The clusters were found to be 10 nm in diameter, and distances between corresponding amino acids of different rhodopsin molecules of the order of 2 nm are not unlikely, especially if these amino acids are located in the C terminus. The lack of additional dipolar broadening

for the sample, in which both sites are labeled, indicates that the distance between site 1 and site 2 exceeds 2.5 nm.

3.5. Conclusion regarding the spin label binding sites

Fig. 5 shows a topographic model of octopus rhodopsin. The transmembrane topology taken from Ovchinnikov et al. [6] was modified according to Hall et al. [15]. The primary structure reveals eight cysteines. Cysteines 109 and 187 most probably form a disulfide bridge, which is found to be of functional significance as the respective cysteines in bovine rhodopsin (Cys110 and Cys187 [16]). Thus they can be excluded as possible labeling sites. Three cysteines are located within the aqueous phase: 337, 338 and 345. Our results, however, strongly suggest that only one of these cysteines is accessible for spin labeling, whereas the neighboring cysteine residues 337 and 338 may be palmitoylated as was found for the corresponding cysteines 322 and 323 of bovine rhodopsin. Therefore site 2 is identical with cysteine residue 345 in the C-terminal region.

Labeled site 1 must be cysteine residue 204, 208 or 220 in helix V. The spin label attached to site 1 was found to be located within the membrane close to the polar head group region. The degree of immobilization suggests a helical environment. The location of the membrane water interfaces with respect to the protein are not known. However, a location of cysteine residue 208 close to the polar head group regions is not likely, which leaves positions 204 and 220 as candidates for site 1.

3.6. Electron paramagnetic resonance spectra change due to photoexcitation

Light-induced changes of the EPR spectra of spin labels attached to octopus rhodopsin were reported by Tsuda and coworkers [17], who used a different kind of spin label. The change in the EPR spectrum was induced by blue light and reversed with orange light. It was concluded that the reversible spectral change corresponds to structural differences of the protein moiety between rhodopsin and metarhodopsin. The question which remained to be answered is where the observed structural change is likely to occur.

Reversible changes of the EPR spectra due to photoexcitation were monitored with our samples after converting rhodopsin to metarhodopsin by blue light (440 nm) (Fig. 6(a)). The difference between the spectra of rhodopsin and metarhodopsin due to photoexcitation shows significant changes in the mobility of the spin label attached to site 2 (Fig. 6(b)). The mobility is increased during the rhodopsin-to-metarhodopsin transition as can be observed from the changes in the low and high field line shapes. The conformational change of the protein causing this signal change is not global, since no spectral changes are detected at site 1. The magnitude of the conformational change in the protein is likely to be small. The change in the EPR spectra is very similar to those observed in the deoxyhemoglobin-to-oxyhemoglobin transition, which involves protein movements in the order of a few ångströms [18,19].

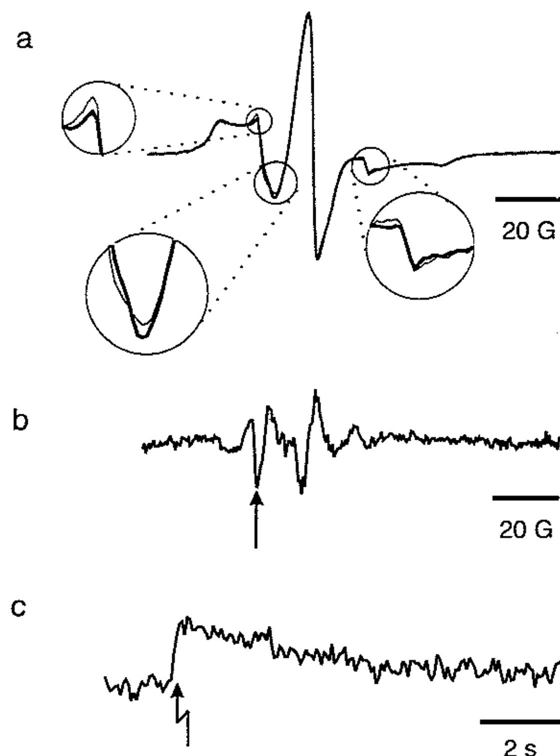

Fig. 6. Curves a, EPR spectrum of spin-labeled octopus rhodopsin (———) and metarhodopsin (———) with the spin label attached mainly to site 2. The low- and high-field regions, where spectral differences are observed, are enlarged. Curve b, EPR difference metarhodopsin – rhodopsin spectrum with the ordinate scale stretched by a factor of ten compared to curve a. Curve c, EPR transient recorded after flash irradiation of metarhodopsin, with same ordinate scale as for curve b. The *B*-field value was fixed at the value corresponding to the negative low field difference peak indicated by the arrow on curve b. The transient shows the EPR spectral changes due to the conversion of metarhodopsin to rhodopsin after a 540 nm laser flash (arrow) and the slow back reaction to metarhodopsin due to continuous irradiation at 440 nm.

The time dependence of the EPR signal at fixed positions of the magnetic field was followed after a 540 nm laser flash during continuous adaption of the sample to light of 440 nm. A superposition of 100 transients is shown in Fig. 6(c). With this irradiation program, the conformational change responsible for the fast EPR spectral changes appears during the transition of metarhodopsin to rhodopsin. The rise of the transient and therefore the underlying conformational change is finished within less than 10 ms and cannot further be resolved owing to the limited sensitivity in the present experiment. As a result of the continuous light adaption, the difference signal disappears with the recovery of the original metarhodopsin fraction. A corresponding transient with the inverse amplitude is observed with the reversed irradiation program. This reversibility shows that the EPR spectral changes correspond to the photoconversion between rhodopsin and metarhodopsin and the formation of other pigments can be excluded. Since the reversibility was reproduced with the same sample over several hours, the contribution of light-

activated enzymes in the photoreceptor membrane is very unlikely. The conformational transition of metarhodopsin to rhodopsin is finished within less than 10 ms at room temperature. This agrees with time-resolved measurements of the absorbance change, which reveal that the metarhodopsin-to-rhodopsin transition is finished within 1.6 ms [20].

What kind of conformational changes could account for the observed EPR spectral changes? The structural change is detected at the cytoplasmic surface of the rhodopsin molecule in the C terminus near helix VII. Motions of this helix or of the C terminus could influence the nitroxide mobility near site 345 and could therefore account for the data. However, the spin label bound at helix V does not sense any changes upon photoexcitation, i.e. the motion of this helix is considered to be less likely.

In summary it can be concluded that two of the eight natural cysteines of octopus rhodopsin are accessible to the spin label MTSSL. One of these spin labels is attached to a cysteine of helix V, whereas the other spin label is bound to Cys345 in the C terminus. A reversible structural change is detected in the vicinity of 345, which occurs during the rhodopsin to meta-
rhodopsin transition and which might be involved in exposing sites for the binding of enzymes required during the transduction process.

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