

LIGHT-INDUCED CONFORMATIONAL CHANGE OF OCTOPUS RHODOPSIN
AS DETECTED BY A SPIN LABEL METHODAkihiro Kusumi,⁺ Shun-ichi Ohnishi, and Motoyuki Tsuda^{*}

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SUMMARY: Using a spin-labeling technique, the light-induced conformational changes in proteins were detected in microvillar membranes of photoreceptor cells of octopus as well as in digitonin extract of the membrane. The change in EPR spectrum of the spin-label was induced with blue light (wavelengths around 420 nm) and the change was reversed with orange light (wavelengths longer than 580 nm). It is concluded that these reversible spectral changes correspond to the differences in the structure of the protein moiety between rhodopsin and metarhodopsin.

When the rhodopsin absorbs a photon, the primary photochemical event is followed by a series of dark intermediates leading to a final photoproduct, metarhodopsin in the case of cephalopod rhodopsin (1). One or more of these processes, meeting the appropriate temporal requirement, lead to visual excitation, which may involve the conformational changes of proteins in photoreceptor membrane (2). It is, therefore, important to clarify what kinds of conformational changes in protein moieties occur during the intermediate processes after illumination.

There have been several studies on the changes in the tertiary structure of the protein moiety of rhodopsin, including measurements of activated molar entropy and volume (3,4), hydrogen exchange (5), diamagnetic anisotropy (6), and saturation transfer EPR (7). However, it would be very desirable to demonstrate that the observed change is reversible when rhodopsin is regenerated, since opsin, the protein part of rhodopsin, is quite a labile protein after photoconversion and is easily denatured.

Octopus rhodopsin is an excellent material for the study of conformational changes upon illumination because the final photoproduct, acid metarhodopsin, is

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stable at physiological temperatures and because rhodopsin can be almost completely photoregenerated from metarhodopsin with orange light (8). This photochemical reversibility at physiological temperatures makes possible experiments which examine the difference in protein structure between rhodopsin and metarhodopsin. Studies of both biochemistry (9,10) and photochemistry (1,3,7,10,12) of the octopus photoreceptor system are being actively pursued.

We utilized the spin-label method in this work, which has allowed us to investigate the local environment around the cysteinyl residues of the proteins. Another advantage of EPR experiments over other spectrophotometric methods is that the measurement is not affected by sample turbidity. This problem is important in studying proteins in membranes because diffraction of light by the sample gives serious artifacts for CD, ORD, and UV spectrophotometries, forcing one to use detergents that, in turn, may perturb the protein structure.

In the present communication, we report direct evidence of the reversible conformational changes of proteins in the photolysis of octopus rhodopsin in the membrane. In order to compare our results with photochemical data, we repeated the spin-label experiment in digitonin solution.

MATERIALS AND METHODS

Microvillar membranes of octopus (*Mizudako*, *Paroctopus defleini*) photoreceptor cells were isolated as described previously (1). After discontinuous sucrose gradient centrifugation, an orange band at the 1.12/1.15 g/ml interface was collected. The pellet was washed three times with water and the loose part of the pellet was collected. This contained the isolated microvillar membranes, which were washed repeatedly with acid (pH 5.7) neutral (pH 7.2), alkaline solution (pH 9.5), and finally with the buffer that was used in the experiment.

The membranes were spin-labeled by the covalent reaction with 4-maleimide-2,2,6,6-tetramethylpiperidinoxyl (MSL) synthesized by the method of Griffith and McConnell (14). Spin-labeling was carried out in a buffer solution (10 mM HEPES, 65 mM NaCl, pH 6.8) containing 1 mM MSL and the membrane ($OD_{476nm} = 0.3$) at 0°C for 2 hr. Unreacted MSL was removed by washing the membranes seven times. In some experiments, the spin-labeled membranes were solubilized with 2% digitonin in 0.1 M acetate-sodium acetate buffer at pH 5.5 for 10 hr. The solution was centrifuged 16,000 x g for 30 min and the clear supernatant was concentrated with Centriflo (CF 25, Amicon Corporation). All the procedures described above were carried out under dim red light.

EPR spectra were obtained on a JEOL FE-2X spectrometer with temperature control accessory. All experiments presented here were carried out at a temperature of 10.0 ± 0.5 C. The samples were irradiated through the cavity

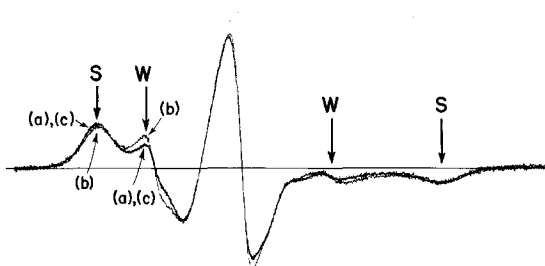


Fig. 1 ESR spectra of spin-labeled microvillar membranes of octopus at pH 6.8. S and W in the figure show the strongly immobilized component and weakly immobilized component, respectively. (a) measured in the dark, (b) after illumination of blue light, (c) after further illumination of orange light. Spectrum (a) and (c) are almost superimposable, so they look like a single thick line. Microwave power of 10 mW and 100 kHz field modulation with the width of 2 gauss were used.

window using a convex lens system. Actinic light was obtained from a 1 KW projector lamp and glass filter (Toshiba VV-42 for blue light and VO-58 for orange light).

RESULTS

It has already been reported that octopus rhodopsin and its acid metarhodopsin can be easily interconverted by alternate irradiation with blue and orange light, because the absorption maximum of acid metarhodopsin (512 nm) is located at a much longer wavelength than that of rhodopsin (476 nm) (*Paroctopus defleini*) (7). Figure 1 shows the EPR spectra of MSL-labeled microvillar membrane of octopus photoreceptor cells. At least two components, a broad peak due to a strongly immobilized signal (s in Fig. 1) and a sharp peak due to a weakly immobilized signal (w in Fig. 1), were observed in the spectra. When the membranes were irradiated with blue light, the height of the broad peak in the spectra decreased and that of the sharp peak increased. This indicates that a fraction of MSL that contributed to the broad peak was converted to that of the sharp peak by irradiation with blue light. This change corresponds to the photoconversion of rhodopsin to acid metarhodopsin. Since the MSL reacted with a cysteinyl residues of proteins, the spectral changes of Fig. 1 indicate a change in local environment around the cysteinyl residues of proteins, which causes less immobilized motion of part of the spin-label. The change in the rotational correlation time between the strongly and the weakly immobilized spin-label mobility is greater than a factor of 100.

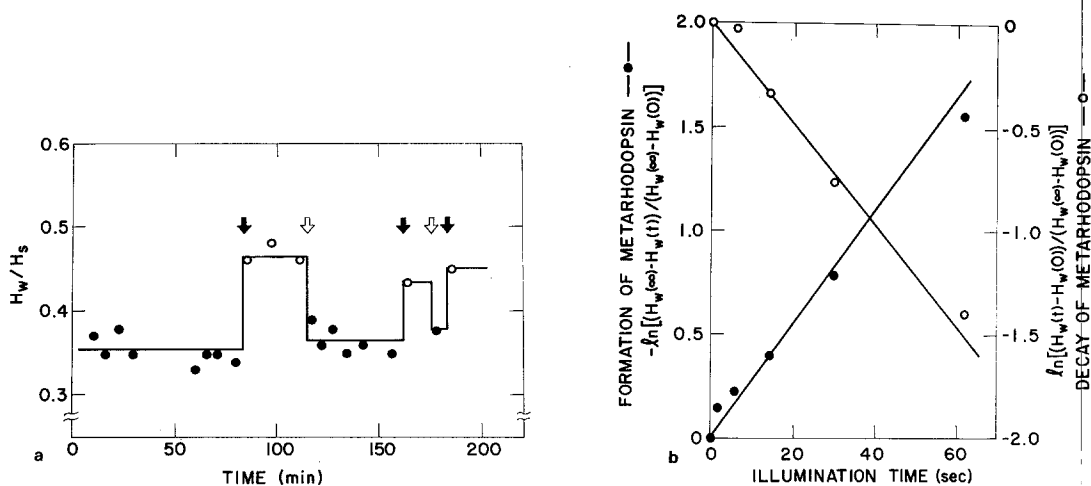


Fig. 2 (a) The effect on H_w/H_s of alternate illumination with blue (↘) and orange (↗) light. Notice that no time-dependent change was observed. (b) The change in H_w plotted against illumination time.

Irradiation of the acid metarhodopsin with orange light reversed the spectral change ((b)(c) in Fig. 1), which is almost the same as the spectrum of rhodopsin. The spectral change was parametrized as a ratio of H_w/H_s , where H_w and H_s represent the peak height of the sharp peak (weekly immobilized MSL) and that of the broad peak (strongly immobilized MSL), respectively.

Figure 2A shows the changes in H_w/H_s with irradiation of blue and orange light. The reversibility of the EPR spectra was almost complete even after the membrane was irradiated alternately with blue and orange light more than 10 times at 10°C . There is no further change in EPR spectra in the dark after the light exposure. This result suggests that changes in the EPR spectra are solely due to the initial pigment and the photoproducts present at the end of each irradiation and not to any thermal decay products with significantly longer lifetimes ($\tau > 1\text{sec}$). Using light of weaker intensity, changes in the EPR spectra were observed after various durations of light exposure. In Fig. 2B, the value of $(H_w(t) - H_w(\infty))$ was plotted on a logarithmic scale versus the time of irradiation, where $H_s(t)$ is the peak height of the sharp peak at t sec of exposure, and $H_w(\infty)$ is that of the final photosteady state. Both blue and orange irradiation

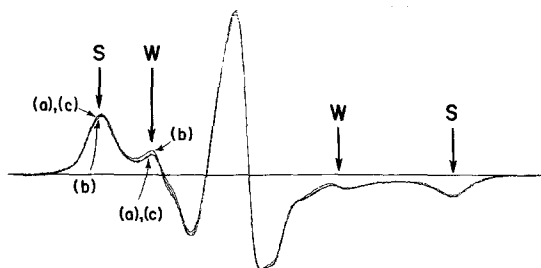


Fig. 3 EPR spectra of digitonin-solubilized membrane. (a) measured in the dark, (b) after illumination of blue light, (c) after further illumination of orange light. Measuring conditions are the same as those in Figure 1.

gave straight lines. This shows that the observed changes in EPR spectra correspond to the photoconversion between rhodopsin and metarhodopsin, with no formation of other pigments.

These results suggest that a local conformational change around cysteinyl residues of proteins takes place in some intermediate between rhodopsin and acid metarhodopsin in the photolysis of octopus rhodopsin.

A similar spectral change was observed in the digitonin-solubilized rhodopsin (Fig. 3). These changes are somewhat smaller than those in the membranes. Spectral features show that the spin-labels are more immobilized than those in the membranes. We did this experiment because most of the photochemical studies have been done in this system.

DISCUSSION

The observed change could either take place in rhodopsin or a protein whose conformation is controlled by rhodopsin. It is most likely that the observed change is caused by the conformational change in the protein moiety of rhodopsin. The reversibility of the spectral change would indicate that the observed changes are associated with rhodopsin or protein that can efficiently interact with rhodopsin. Light-activated enzymes in photoreceptor membranes (GTPase (9)) have been shown to lose their activity very fast (within 10 minutes in the dark (Fig. 2A)). The observation of the light-induced change when the pigment has been solubilized in detergent, which may uncouple any proteins controlled by rhodopsin, also suggests that the observed change is a direct reflection of the

conformational change in rhodopsin itself. The linear relationship in Fig. 2B suggests that this conformational change takes place only in a photoconversion process between rhodopsin and acid metarhodopsin, and not between other pigments.

There have been a number of studies that attempted to measure the conformational change of the protein moiety of rhodopsin. But most of them have been carried out in the presence of detergents (15-17). We think it is advantageous to study the structure of integral membrane proteins in the membrane and not in the presence of detergents since detergents could seriously perturb the tertiary structure of proteins. Shichi et al. (18), for example, reported that α -helix content of bovine rhodopsin in digitonin changed upon illumination, while such changes could not be found in the rod outer segment membranes. This result suggests that the light-induced conformational change in digitonin-solubilized rhodopsin is a result of perturbation caused by the detergent.

The spin-labeling method gives us a reliable and easy way to detect the conformational change of a membrane protein in situ. It is to be noticed that the spectral changes are reversible, which strongly suggests that these changes are not artifacts, due to such things as protein denaturation or raised temperature.

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