Stability and Regeneration of Rhodopsin Absorption Spectra at an Air-Water Interface

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Stability and regenerability of the absorption spectrum of CTAB solubilized rhodopsin at an air-water interface is studied. The spectral properties of rhodopsin films in the dark are stable more than 150 minutes. When rhodopsin is bleached (40–50%) and maintained in highly compressed films recovery of the spectrum is observed. The recovery is 57% to 100% of the pigment present before irradiation. At low surface pressure or if the films are expanded after irradiation there is no observable recovery.

Introduction

Reaction associated with visual disc membranes are investigated using monolayer techniques. Detergent solubilized rhodopsin is reconstituted into films at an air-water interface. The spectral characteristics and transformations of these films are used as a model for the *in vivo* processes. The surface properties of fragments of outer rod segments at an air-water interface were reported in a previous paper [1].

It is necessary to determine what effects the preparative procedure has on the native integrity of rhodopsin. The phospholipid and protein conformation might be altered by certain detergents. Hong and Hubbell [2–4] suggested that rhodopsin might exist in various states of partial denaturation even though the characteristic absorption spectrum at 500 nm is maintained.

In addition to the purification problem, there always is the question of whether or not, or to what extent, denaturation occurs when protein films are formed at an interfacial system.

In this work, two criteria are used to evaluate the molecular integrity of our rhodopsin preparations at the interface: first the presence and stability of the rhodopsin absorption spectrum in the dark and second the regenerability of rhodopsin after photolysis. It has been reported [3, 5, 6] that phospholipid is necessary to regenerate rhodopsin. Hubbell [4] proposed that the regeneration of bleached rhodopsin may depend on the protein environment rather than phospholipid content. The latter is determined by the type of detergent used to extract rhodopsin. The protein environment actually consists of both chemical and physical aspects. We have approached the latter aspect by forming rhodopsin into a film at an air-water interface. The surface pressure, pigment-pigment and pigment-lipid interaction can be varied by using mixed films and compressing the film. As an initial step in considering the effect of detergent treatments, we have examined CTAB (cetyl trimethyl-ammonium bromide) solubilized rhodopsin.

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Materials and Methods

The procedures for preparing rod outer segments (ROS) are essentially the same as described previously [1]. The main difference is a further purification using sucrose density gradient centrifugation. Linear sucrose gradients (density 1.12 to 1.18) in 66 mM phosphate buffer (PB) are prepared and the ROS-PB suspension is layered on the sucrose gradient. The samples are centrifuged at $100{,}000 \times g$ for 30 min. The top dark red band is collected and washed with PB by centrifuging the sample at $48{,}000 \times g$ for 15 min. The pellet is solubilized in 10–15 ml of 1% CTAB in 47.6% sucrose solution and mixed at 5 °C for about 1 h. After mixing, the solution is again centrifuged at $48{,}000 \times g$ for 20 min, to obtain a clear, bright red supernatuant. All preparation steps and experiments are performed in a dark room with deep red light (Kodak No. 2 red filter in combination with 25W G.E. red safe light). Temperatures of materials are maintained at 5 °C throughout the preparation.

The optical density (O.D.) of ROS and the detergent solubilized rhodopsin are measured using a Cary (Model 14R) spectrophotometer. The spectrum is characteristic of rhodopsin as published by

other workers [7]. The purity of rhodopsin used for this work is assayed spectrophotometrically from the ratio $R_1 = A_{280}/A_{500}$, and $R_2 = A_{400}/A_{500}$, where A_{280}, A_{400} and A_{500} represent the O.D. at 280, 400 and 500 nm, respectively. In our preparation $R_1 =$ 1.9 to 2.1 and $R_2 = 0.21$ to 0.31. For comparison purposes the ratios $R_1 = 1.75$ and $R_2 = 0.21$ are reported for digitonin solubilized rhodopsin [7].

Details of the film balance used to measure surface properties and of the optical system to measure absorption spectra of rhodopsin films at an air-water interface were reported previously [1, 8, 9]. A Perkin-Elmer autobalance (Model AM-2) is used to measure the surface pressure measurements of films. A 1% CTAB-rhodopsin film is formed on the subphase (PB, pH 7.0 ionic strength 0.066). Approximately 4.3×10^{15} rhodopsin molecules are added to the surface in 300 to 400 μl of solution. The temperature of the subphase is thermostatically maintained at 15 °C. The light source used for photolysis is an unfiltered, low pressure, mercury arc lamp (2×10^3 erg/cm^2 sec).

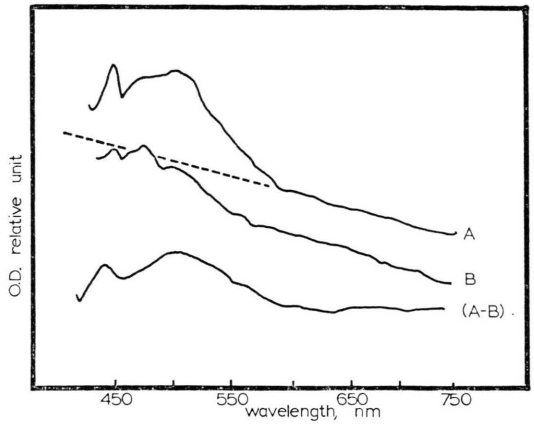

Fig. 1. Absorption spectra of a film of CTAB solubilized rhodopsin at an air-water interface. Surface pressure of the film is maintained at 40 dyn/cm. Curve A is the spectrum before illumination. Curve B is the spectrum after 6 min illumination. Curve (A−B) is the difference in absorption between the two curves. The intensity of the unfiltered, low pressure Hg light on the film is 3.8×10^5 erg/cm^2 sec. The subphase contained phosphate buffer, pH 7.0 and was maintained at 15 °C throughout the experiment.

Results and Discussion

Absorption spectra of rhodopsin films at an air-water interface (uncorrected for scattering) before and after illumination are shown in Fig. 1. Before irradiation of rhodopsin films maxima are observed at 448 and 497 nm. Frequently in our data, it appears that the latter band may consist of two maxima, one at 472 and another at 502 nm. After irradiation for 6 min there is a general decrease in absorption. The difference between the absorption spectrum in the dark and the spectrum after illumination is shown as curve (A − B) in Fig. 1. Maxima are observed at 452 and 513 nm. On the basis of correlations of spectral data the absorption maxima observed in Fig. 1 might correspond to various intermediates in the bleaching of rhodopsin. In the case of rhodopsin films the trapping of intermediates might arise from a modification of the activation energy induced by the highly compressed film.

To correct the absorption spectrum a bleached film is used as a baseline. We have also used an alternative procedure that gave almost identical results. In this latter procedure the linear sloping "spectrum" observed with films between 750 and 600 nm may be safely assumed to arise from scattering by the film as there is no significant absorption by rhodopsin in this region. Assuming the scattering may be approximated by a linear relationship, the scattering determined between 750 and 800 nm is extrapolated to 400 nm (see dotted line in Fig. 1).

When rhodopsin is bleached at low film pressure (15 dyn/cm) there is no spectral recovery. In fact if films irradiated in the compressed state, are expanded and recompressed in the dark, there is no observable recovery of the absorption spectrum. In order to have recovery the films must be irradiated and maintained in a highly compressed state.

The stability of the absorbance by rhodopsin film, in the dark was measured as a function of time (Fig. 2). For a film compressed to 40 dyn/cm the optical density at 500 nm (O.D. 500) remains constant for over two hours. The absorption coefficient $E_m = \text{O.D.} \times A$, where A is the area/molecule and O.D. is the optical density per monolayer. The resulting $E_m = 4.9 \times 10^4$ mol/cm. Irradiation for 2 min produces no significant bleaching, but an additional 4 min irradiation results in a 30% decrease in O.D. 500.

In order to make accurate measurements of absorption spectra, relatively high absorbances are required for the rhodopsin films. This is accomplished by maintaining a high surface pressure,

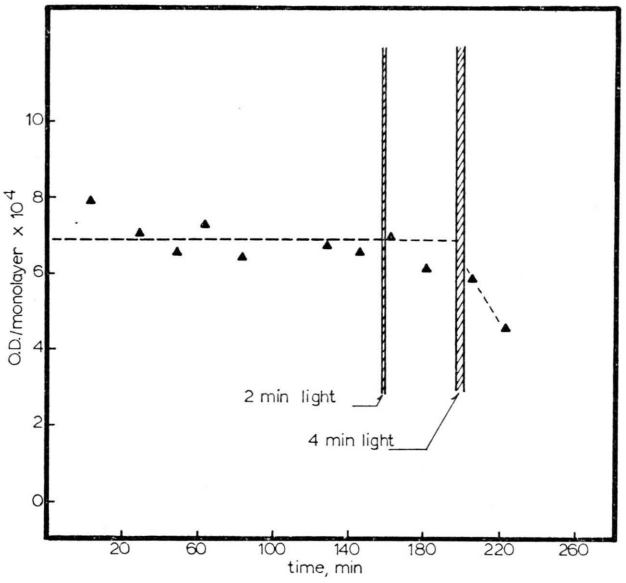

Fig. 2. The optical density/monolayer at 500 nm, of CTAB solubilized rhodopsin at an air-water interface as a function of time. The O.D. remains constant in the dark for over 150 min. A 2 min illumination period has no significant effect on the O.D. After another 4 min illumination a decrease in O.D. is observed. Surface pressure of the film is 40 dyn/cm. Light intensity and subphase conditions as given in Fig. 1.

thereby obtaining a high pigment concentration on the surface comparable to what is obtained *in vivo*. Assuming that rhodopsin forms a monomolecular film, the area/molecule is calculated to be 670 Å^2, this is equivalent to a three dimensional concentration of 0.09 M.

Regeneration of the absorption spectrum of rhodopsin in a compressed film at an air-water interface is observed. In Fig. 3 is shown the time course of O.D. 500. A rhodopsin film is irradiated for two, 4 min periods during the course of an experiment. Recovery of O.D. 500 is 57% after the 1st photolysis and after the 2nd photolysis about 100% of the O.D. 500 remaining after the 1st photolysis. Recovery after each irradiation takes about an hour. The spectrum of the recovered pigment is similar to the original unbleached spectrum. The percent recovery is calculated using the following relationship:

$$\left[\frac{(A_{500}, \mathrm{L}, \mathrm{d}) - (A_{500}, \mathrm{L})}{(A_{500}, \mathrm{d}) - (A_{500}, \mathrm{L})}\right]$$

where (A_{500}, d) is the absorbance at 500 nm in dark before each photolysis, (A_{500}, L) is the absorbance after a photolysis (the minimum after a light period in Fig. 3) and $(A_{500}, \mathrm{L}, \mathrm{d})$ is the absorbance in the dark period after a photolysis (the maximum reached in the dark after a light period in Fig. 3). When irradiation is carried out for 30 min, absorbance decreases more than 90% and almost no recovery is observed (Fig. 3).

In a parallel experiment, with a solution of CTAB solubilized rhodopsin, 5 min of light resulted in 75% bleaching. After half an hour in dark there is only about 20% recovery of absorption at 500 nm.

We have observed that the spectral properties of films of CTAB extracted rhodopsin is both stable

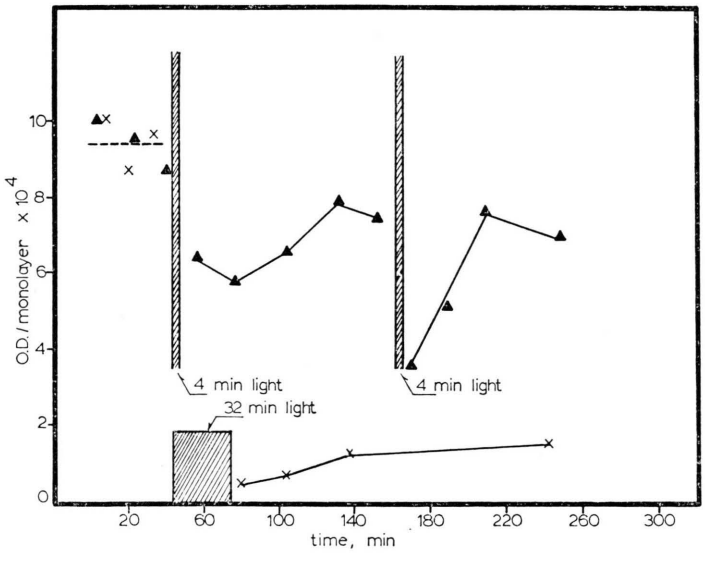

Fig. 3. Optical density/monolayer at 500 nm, of CTAB solubilized rhodopsin, as a function of time and illumination. The bleaching and regeneration of rhodopsin with 4 min illumination periods is shown by triangles (▲ — ▲). After the first 4 min light there is 75% recovery. After the second 4 min light there is close to 100% recovery. The "irreversible" bleaching of rhodopsin after 32 min of illumination is shown by crosses (× — ×). Illumination periods are shown by cross hatched areas. Surface pressure is 30 dyn/cm. Light intensity and subphase conditions as given in Fig. 1.

at the air-water interface and undergoes reversible bleaching. These results indicate that CTAB solubilized rhodopsin does not significantly disturb the native chemical and physical environment of the protein at an air-water interface. Most significant is that regeneration of the absorption spectrum of rhodopsin in compressed films occurs without the addition of cis retinal, phospholipid or isomerase. This is in sharp contrast with regeneration studies of rhodopsin in solution where the addition of 11-*cis* retinal or isomerase is required [3, 6].

Korenbrot and Pramik [10] reported on the formation of mixed films of lipid and rhodopsin (mole ratio 150 : 1) on both an air-water interface and deposited as multilayers on glass. When air dried rhodopsin containing multilayers are illuminated a stable meta I (480 nm) is formed. This form can be fully bleached by increasing the relative humidity or photoconverted into rhodopsin and presumably isorhodopsin. They reported chemical regeneration of rhodopsin at an air-water interface is dependent on the presence of 11-*cis* retinal dissolved in the subphase. The spectroscopic properties of rhodopsin regenerated in the interfacial films are indistinguishable from rhodopsin in intact disc membranes. The work reported here differs in several important respects from that of Korenbrot and Pramik. Our rhodopsin films do not contain large proportions of lipid, as no lipid is added to the rhodopsin preparation. Furthermore, no 11-*cis* retinal is added to the system. The orientation of rhodopsin at an air-water interface is probably altered when deposited in multilayers on a glass slide. Also the pigment-lipid areas are not well defined in the multilayers prepared by Korenbrot and Pramik since surface pressure was not held constant and deposition ratios were not measured.

There are a few factors that could contribute to the spectral stability, bleaching and regeneration of our rhodopsin films. For example, the CTAB solubilized rhodopsin may still have some lipid components which aid in stabilizing the spectral properties on the water surface. In addition, the rhodopsin transformations in a film may be regulated by the physical parameters imposed by the orientation and close molecular packing at the high surface pressure. The high film pressure might restrict gross changes in protein conformation that can ordinarily occur at low film pressure or in solution. From our preliminary data it appears that there is a relationship between the surface pressure of the film, the ability to regenerate the rhodopsin spectrum and the percent bleaching per minute of illumination.

These observations indicate that CTAB solubilized rhodopsin in highly compressed films at a gas-water interface might carry out reactions similar to those observed in disc membranes. This work was supported, in part, by N.I.H. grant RO-1-EY00173.

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