Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 853-859

ON THE CORRELATION BETWEEN LIGHT-INDUCED PROTEIN FLUORESCENCE CHANGES AND THE FORMATION OF METARHODOPSIN III$_{465}$ IN BOVINE PHOTORECEPTOR DISK MEMBRANES

Tsukasa Chiba, Hiroshi Asai and Hideo Suzuki

Department of Physics, Waseda University
Tokyo 160, Japan

Received December 26,1979

Summary: The correlation between the absorption spectral changes and the increase in protein fluorescence after short illumination of suspensions of bovine photoreceptor disk membrane fragments was investigated. A comparison of the kinetics of the thermal formation of rhodopsin photoproducts with those of the increase in fluorescence indicates a close correspondence between the thermal formation of metarhodopsin III$_{465}$ and the light-induced fluorescence increase. This result suggests that a conformational change, probably involving a decrease in the polarity of the environment of tryptophan residues, occurs in association with the formation of metarhodopsin III$_{465}$.

Introduction: After the absorption of photons, the bovine visual chromoprotein, bovine rhodopsin, is hydrolyzed into an apoprotein (opsin) and all-*trans* retinal through several photointermediate states. Studies on the identity of these photointermediates have been described in a review paper by Wald (1). On the other hand, we have reported in a previous paper that the turbidity of aqueous suspensions of bovine rod photoreceptor disk membranes, measured at a wavelength of 800 nm, decreases by about 2% after photobleaching (2). Furthermore, we have observed that the protein fluorescence due to tryptophan residues increases slowly in the dark after short illumination, and suggested that this change might be related to the process of adaptation (3).

In the present study, we have attempted to identify the photointermediate state in bovine photoreceptor disk membrane which is involved in the slow bleach-induced fluorescence and turbidity changes.

Materials and Methods: Rod outer segment (ROS) disk membrane fragments were prepared according to the method of Chiba et al. (3).

0006-291X/80/030853-07$01.00/0

For the measurements of absorbance and fluorescence, purified ROS disk membranes were suspended (0.25 mg of protein per ml) in 115 mM NaCl, 5 mM KCl and 50 mM N-2-hydroxyethylpiperazine-N´-ethanesulfonic acid (HEPES) buffer (pH 7.5). The samples were illuminated for 5 sec with a 300 W xenon lamp (Varian) through a heat absorbing filter (Hoya Corp., HA-50) and an interference filter (Nippon Shinku, λ_{max} = 563 nm). A Shimadzu recording spectrophotometer (model UV-350) attached to an integrating sphere was used for measurements of absorbance. Absorption spectra were taken at appropriate intervals with a scanning speed of 100 nm/min. A double-beam difference spectrofluorophotometer (Shimadzu model RF-503) was used for measurements of fluorescence. The time course of fluorescence change at 338 nm (the monochromator bandwidth was 4 nm) was measured with excitation at 290 nm (the monochromator bandwidth was 5 nm). The temperature of cells was controlled to ±0.5°C using a Taiyo Coolnit CL-15 circulating water system.

Results and Discussion: It was observed that the absorption spectrum of a suspension of bovine ROS disk membrane fragments changed gradually after a short illumination of the suspension (Curves 2-6 in Fig. 1a). After the end of scanning of curve 6, the absorption spectrum remained unchanged for at least 15 min. Curve 7 in Fig. 1a was obtained after the addition of 20 mg of hydroxylamine (the final concentration was 100 mM) to the sample 40 min after bleaching. The amount of unbleached rhodopsin was estimated from the absorbance at 498 nm of this spectrum. Addition of hydroxylamine caused no change of the absorption spectrum of unbleached rhodopsin, and retinal oxime was formed from all-trans retinal of bleached rhodopsin. The absorption maximum of retinal oxime was 367 nm (Curve 7 in Fig. 1a) and the absorbance of retinal oxime was nearly zero at wavelengths longer than 480 nm. Therefore, by comparison with the spectrum of unbleached rhodopsin in its initial state (Curve 1 in Fig. 1a), we could estimate the absorption spectrum of the rhodopsin remaining in the unbleached state (Curve 8 in Fig. 1a). The changes of absorption spectra in the dark after illumination were made clearer by the subtraction of curve 8 in Fig. 1a from curves 2-6 in Fig. 1a (Curves 2-6 in Fig. 1b). The absorption spectrum changes in Fig. 1b show the conversion of

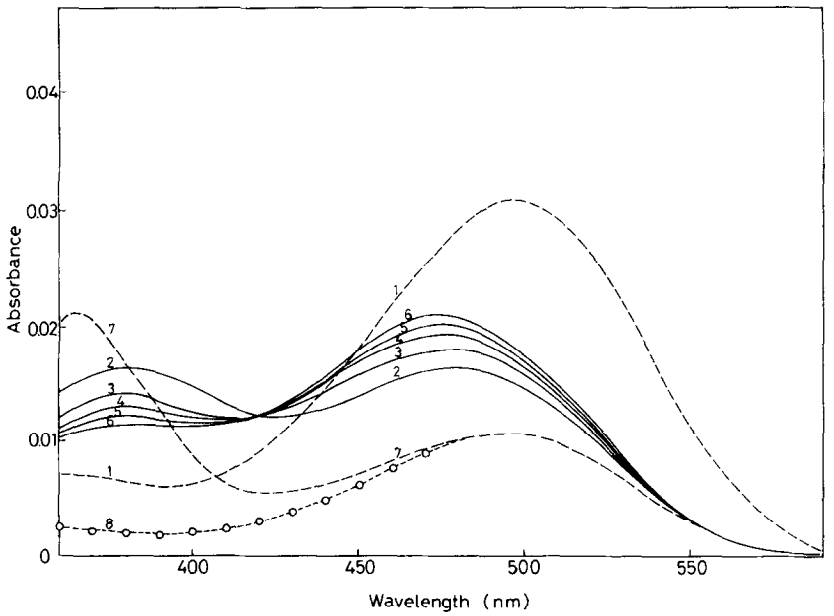

Fig. 1a: Spectral changes at pH 7.5, 22°C. The scanning speed was 100 nm/min. Spectrum 1 was taken before illumination. Spectrum 2 was started at 600 nm, 10 sec after the end of the illumination. Spectrum 3, 3 min 10 sec; Spectrum 4, 6 min 10 sec; Spectrum 5, 11 min 10 sec; and Spectrum 6, 19 min 10 sec after the end of the illumination. Spectrum 7 was taken after the addition of hydroxylamine. Spectrum 8 is the corrected spectrum of unbleached rhodopsin remaining after illumination. The extent of bleaching was about 66%.

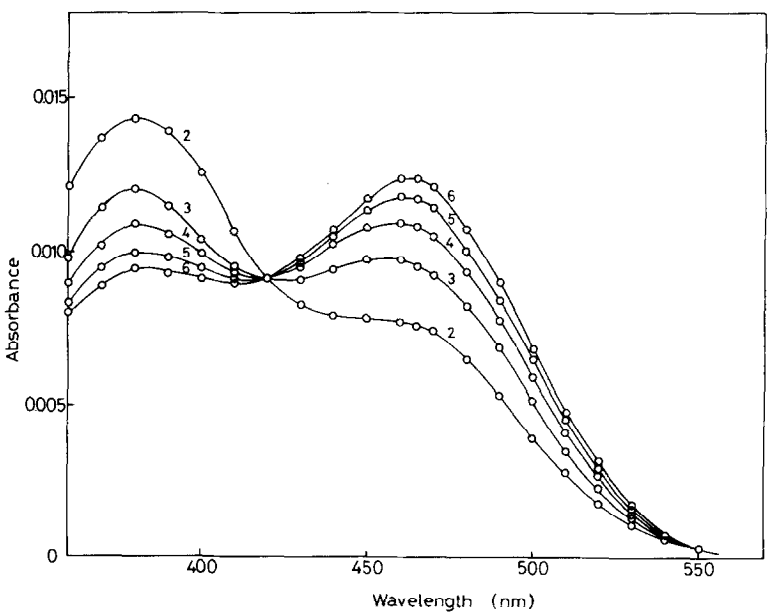

Fig. 1b: Corrected spectral changes after illumination for 5 sec. Spectrum 2 was started at 600 nm, 10 sec after the end of the illumination. Spectrum 3, 3 min 10 sec; Spectrum 4, 6 min 10 sec; Spectrum 5, 11 min 10 sec; and Spectrum 6, 19 min 10 sec after the end of the illumination.

metarhodopsin II_{380} (Meta II_{380}) to metarhodopsin III_{465} (Meta III_{465}). Under the slightly alkaline conditions that we used (pH 7.5), large amounts of Meta III_{465} were accumulated (Curve 6 in Fig. 1b). A similar accumulation of Meta III_{465} was also reported by Gyllenberg et al. in frog retina (4). The absorption spectrum of Meta II_{380} is similar to that of all-trans retinal$_{387}$ (5), so that the direct conversion of Meta II_{380} to all-trans retinal cannot be detected spectrally. Since we used an interference filter (λ_{max} = 563 nm) for illumination, the generated Meta II_{380} and Meta III_{465} did not reabsorb light at 563 nm (see Fig. 1b).

The time courses of absorption change at 465 nm (the thermal formation of Meta III_{465}) and at 380 nm (the thermal decay of Meta II_{380}) are shown in Fig. 2-(a),(b). The time course of protein fluorescence change is also shown in Fig. 2-(c). As Guzzo observed in frog retina (6), Fig. 2-(c) shows a rapid decrease in protein fluorescence intensity, followed by a gradual increase. In Fig. 2, it can be seen that the fast decrease in fluorescence intensity is generated in association with the formation of Meta II_{380}, and that the subsequent increase in fluorescence intensity is generated with the formation of Meta III_{465}. Furthermore, in the primary study at low temperature (4-6°C), no fluorescence change was observed on illumination, in spite of the formation of metarhodopsin I_{478} (Meta I_{478}) which was observed spectrally. This means that the intensity of fluorescence from the Meta I_{478} state was nearly equal to that of unbleached rhodopsin (Chiba et al., in preparation).

The kinetics of formation of Meta III_{465} from Meta II_{380} were analyzed, assuming a first order process, and compared with the kinetics of the protein fluorescence increase. The following equation was obtained for the formation of Meta III_{465}.

$$\ln \frac{A(t)-A(\infty)}{A(0)-A(\infty)} = -kt,$$

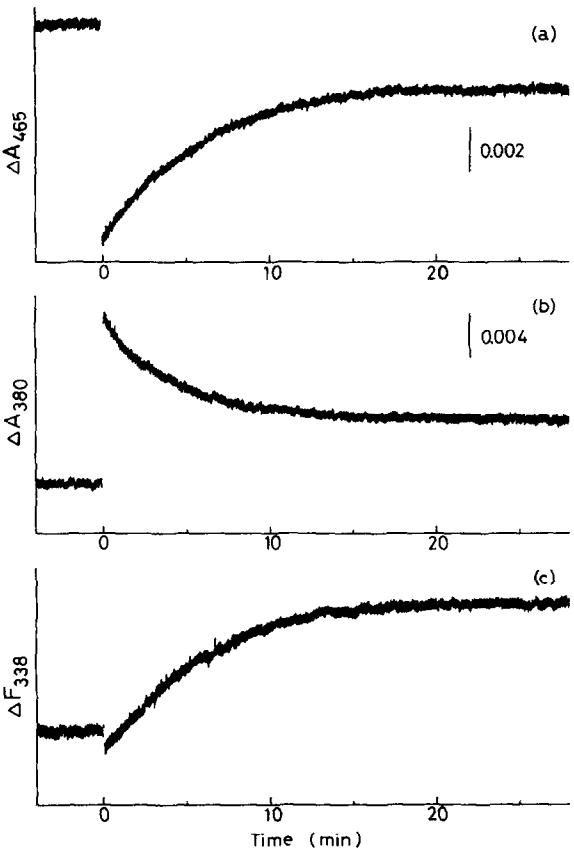

Fig. 2: Time courses of absorbance change at 465 nm (a), and at 380 nm (b) measured at 22°C. The time course of fluorescence change at 338 nm with excitation at 290 nm, measured at 22°C, is also shown (c). Illumination was started at time zero for 5 sec.

where A(0), A(t), and A(∞) are the absorbances at 465 nm at time zero, time t, and infinite time, respectively. From Fig. 2 (a), (b),(c), the first order rate constants were estimated to be 2.9 x 10^{-3}, 3.6 x 10^{-3}, 2.9 x 10^{-3} (sec^{-1}), respectively. The variation of rate constants was within the range of experimental error (see also Fig. 3). The kinetics of the turbidity changes were also the same as those of the formation of Meta III_{465}.

The temperature dependences of the time courses of absorbance change at 465, and 380 nm were examined, as well as that of the fluorescence change at 338 nm. Since the generated Meta III_{465} was stable even at temperatures higher than 30°C for at least 10

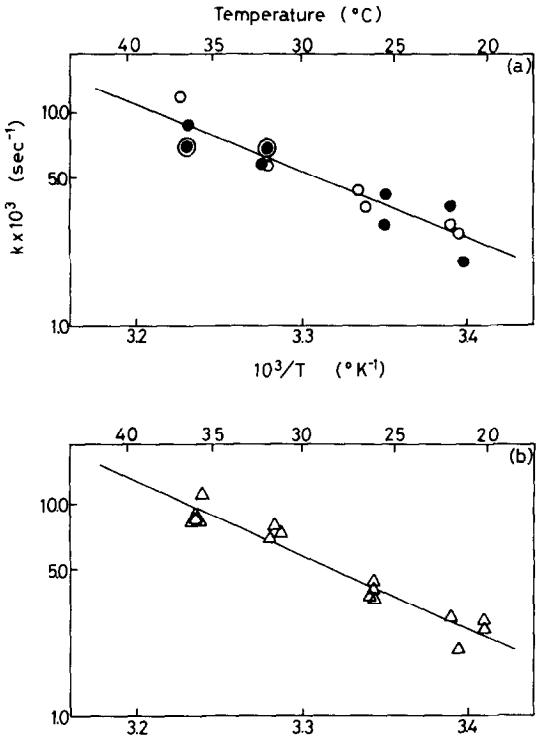

Fig. 3: Arrhenius plots of k for absorbance change (a) and for fluorescence change (b). Rate constants were obtained from the time courses of absorbance change at 465 nm (o) and 380 nm (●) and of fluorescence change at 338 nm (Δ).

min after bleaching, we could estimate the rate constants from the data within this time range. Arrhenius plots of k are shown in Fig. 3 (a) and (b) for absorbance and fluorescence, respectively. The activation energies calculated from the slopes of the lines in Fig. 3 are 14.2±2.8 kcal/mole for the formation of Meta III_{465} and 15.6±2.0 kcal/mole for the increase in protein fluorescence. It is concluded that the light-induced protein fluorescence increase in ROS disk membrane fragments corresponds to the formation of Meta III_{465}.

It would seem that Meta III_{465} is a significant species in the regeneration of native rhodopsin by light. It has been reported that regeneration by light does occur more easily from Meta III_{465} than from Meta II_{380} (7).

We suggest that the thermal formation of Meta III_{465} is associated

with conformational changes of the protein part of rhodopsin, involving a decrease in the polarity of the environment of tryptophan residues of rhodopsin. Furthermore, this process may be related to the regeneration by light, or to adaptation.

References:

1. Wald, G. (1968) Science 162, 230-239
2. Asai, H., Chiba, T., Kimura, S. and Takagi, M. (1975) Exp. Eye Res. 21, 259-267
3. Chiba, T., Asai, H. and Suzuki, H. (1978) Biochem. Biophys. Res. Commun. 85, 551-557
4. Gyllenberg, G., Reuter, T. and Sippel, H. (1974) Vision Res. 14, 1349-1357
5. Donner, K.O. and Reuter, T. (1969) Vision Res. 9, 815-847
6. Guzzo, A.V. (1973) Vision Res. 13, 2581-2586
7. Reuter, T. (1976) Vision Res. 16, 909-917