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EVIDENCE THAT A LIGHT-ACCELERATED ABRUPT CHANGE IN MEMBRANE CHARACTERISTICS OF BOVINE ROD OUTER SEGMENT FRAGMENTS TAKES PLACE AT ACIDIC PH

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Summary: Changes in the turbidity of suspensions of bovine rod outer segment fragments induced by rhodopsin bleaching were measured in the presence of various concentrations of divalent cations at acidic pH (4.7-5.4). Unlike the situation at neutral pH, the turbidity of the suspensions increased drastically by bleaching at acidic pH. It was found that the extent of turbidity change became maximum at a particular concentration of divalent cations (i.e., 5 mM $CaCl_2$, 5 mM $MgCl_2$, or 5 mM mixed divalent cations). However, the turbidity increment in the presence of 5 mM $MgCl_2$ was greatly enhanced by the addition of a minute amount of $CaCl_2$. These results evidently show that the membrane characteristic is abruptly changed by bleaching at acidic pH in particular. It is also suggested that there are two kinds of binding sites for Ca ions: one is a Ca^{2+} specific site, and the other is a nonspecific site to which Mg^{2+} can also bind.

Introduction: The primary process in the visual excitation of a vertebrate photoreceptor is the absorption of photons by the visual pigment, rhodopsin; a series of physical and chemical reactions occur subsequently in the photoreceptor cell. The absorption of one photon in a disc membrane probably gives rise to a change in an electrical potential of the photoreceptor cell. However, the precise mechanism of visual excitation is poorly understood.

It has been reported that when rhodopsin is bleached, calcium ions may migrate from the disc membranes to the plasmalemma of the receptor outer segment, where Ca ions presumably block the dark conduction of sodium ions, resulting in hyperpolarization of the plasmalemma (1,2). Studies of the osmotic swelling and shrinking of isolated rod outer segments also indicate that light reduces the sodium permeability of the plasmalemma (3,4). McConnell (5) suggested that the light-induced proton uptake in bovine rod outer segment fragments is closely related to pH and solute conditions, and secondarily to the

Abbreviations: ROS, rod outer segment; NRO_{440}, N-retinylidene-$opsin_{440}$.

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volume of the discs. Therefore, it is important to investigate the conformational changes of the disc membrane by rhodopsin bleaching. In a previous report (6), the turbidity by bleaching was found to decrease about 2 % at neutral pH. In this paper, the turbidity of unbleached and of bleached suspensions was measured in detail at acidic pH.

Materials and Methods: The rod outer segment (ROS) fragments were prepared as described in the previous paper (7). The purified suspension of ROS fragments was stored in 10 mM Tris-HCl buffer solution (pH 7.4) at 0° C until use. All operations were carried out under dim red light at 4 °C.

The experiments were carried out as follows. First, 0.2 ml of the stored suspension of ROS fragments was diluted with 2.8 ml of a solution containing sucrose, divalent cations, and acetic acid, and the diluted suspension was immediately stirred. The solution conditions of diluted suspensions are indicated in the text or figure legends. About 2 min after diluting the stored suspension (to 0.05-0.1 mg of protein per ml) the turbidity of the suspension at 700 nm was measured in a spectrophotometer. Twelve minutes after the preparation of a diluted suspension, the ROS fragments were illuminated for 2 min at a distance of 15 cm from a 15-W fluorescent white light or a 40-W tungsten lamp through an interference filter (λ_{max} = 550 nm). The percent change in turbidity by rhodopsin bleaching was evaluated by the measured turbidity of unbleached and of bleached suspensions at 14 min after their preparations. The amount of the percent change in turbidity by bleaching with the tungsten lamp through an interference filter was equal to that with the fluorescent white light.

Turbidity measurements of the suspension were carried out at 13° C with a Hitachi 356 spectrophotometer using a head-on type photomultiplier. The distance between the photomultiplier and the optical cell was 33 cm. The optical absorption was nearly zero over the range of wavelength from 660 nm to 880 nm. When turbidity changes were measured at the wavelength of 700 nm during illumination of samples by a white light or a monochromatic light, a cut-off filter transparent at 700 nm was inserted in front of the photomultiplier for its protection. Wavelength dependence of turbidity was measured with a Hitachi 557 spectrophotometer.

Results and Discussion: Fig. 1 shows changes in the turbidity of ROS fragment suspensions with time. Before illumination, the turbidity of a suspension containing 100 mM sucrose, 5 mM $CaCl_2$ and 0.1 % (V/V) acetic acid (pH 5.0) increased gradually and slightly with time. By bleaching, the turbidity increased markedly and abruptly from 0.18 to 0.245; the increase was about 41 %. On the other hand, the turbidity of a suspension which was not illuminated continued to increase slightly. Wavelength dependence of turbidity in a suspension is an indicative of size of the suspended particles (8). The slope that the logarithm of the turbidity of ROS suspension was plotted against the logarithm of wavelengths, was nearly 0.90 at 12 min in the dark after diluting and nearly 0.73 after illumination. Light scattering theory of particles suggests that an

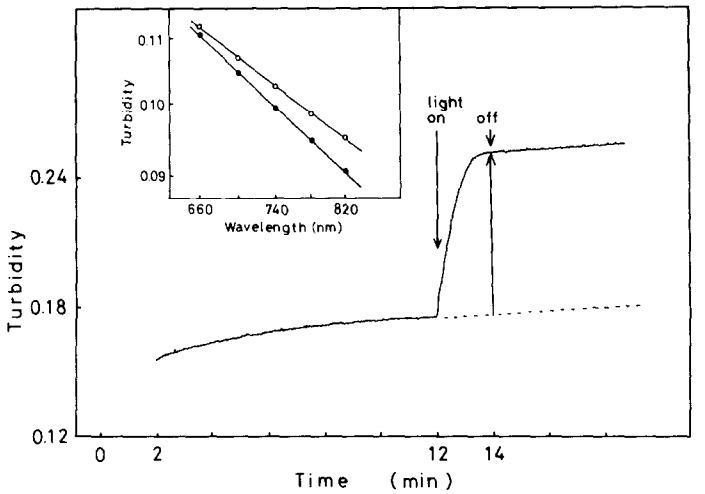

Fig. 1: Tracings of records of the turbidity of ROS suspensions at 700 nm against the time after the preparation of a diluted suspension. On illumination for 2 min, the turbidity increased sharply (the solid curve). In the absence of illumination the turbidity increased slightly (the dotted curve). In the inset, logarithm of turbidity was plotted against logarithm of wavelength. The scanning speed was 300 nm/min. •, Unbleached; ○, bleached. Conditions after dilution; 100 mM sucrose, 5 mM $CaCl_2$ and 0.1 % (V/V) acetic acid (pH 5.0).

increase in turbidity of a suspension of particles corresponds to a shrinkage of the particle size or aggregation of the particles. Mie theory or its extension suggests that turbidity is closely related to the change of the phase of a light-ray passing through a particle, namely, $4\pi a(m-1)/\lambda$, where m is relative refractive index of the particle and a is the radius of the gyration of the particles (8). It seems, therefore, that abrupt increase in turbidity of ROS fragment suspension observed is due to the shrinkage of the membrane fragment and most probably its aggregation.

The pH-dependence of the turbidity of ROS suspensions was investigated over the range of pH from 5.4 to 4.7 (adjusted with HCl or NaOH) and at pH 7.4. As the pH was decreased, the turbidity of unbleached suspension as well as that of bleached suspension increased. The maximum increase in turbidity by bleaching was about 40 % at pH 4.95 (see Fig. 2). In agreement with the previous results (6), the decrease in turbidity at neutral pH by bleaching was about 2 %. The pH at which no light-accelerated turbidity change occurred could not be determined precisely, but appeared to be in the vicinity of pH 6. It has been

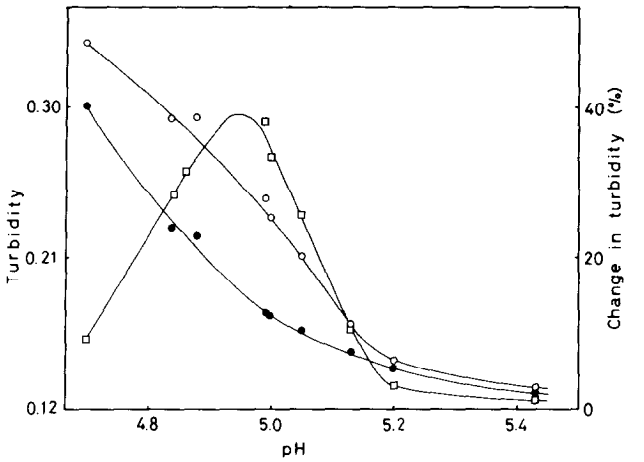

Fig. 2: The pH dependence of the turbidity of ROS suspensions containing 100 mM sucrose, 5 mM $CaCl_2$ and 0.1 % (V/V) acetic acid (pH 5.0); turbidity of (●) unbleached and of (○) bleached suspensions, (□) the percent increase of the turbidity by bleaching. The pH was adjusted with HCl or NaOH. Turbidity was measured at 700 nm.

reported by Ostroy (9) that metarhodopsin II_{380} thermally decayed to N-retinylidene-opsin$_{440}$ (NRO_{440}) at pH values below 5.4 and to a mixture of NRO_{440} and metarhodopsin III_{465} at pH 5.4-6.0. Bennett (10) reported that metarhodopsin II_{380} is shown to be diprotonated at 3° C, and to exist in at least two forms at higher temperature: the diprotonated form observed at 3° C (mainly at acid pH), and an unprotonated form (mainly at alkaline pH). In our experiments, we observed that the percent changes in turbidity of unbleached and of bleached suspensions increased abruptly at pH below 5.2, and were small at pH above 5.2. Therefore, the light-accelerated turbidity change may be related to formation of photointermediates such as the diprotonated metarhodopsin II_{380} or NRO_{440}, and to the character of the membrane. Thus, the diprotonated form of metarhodopsin II_{380} or NRO_{440} may play an important role in the primary process of photoreception. Furthermore, the change in membrane characteristics observed at acidic pH in our experiments may be biologically significant, since hydrogen ions for diprotonation of metarhodopsin II_{380} are clearly much more abundant at acidic pH than at neutral pH.

Fig. 3 shows the relationship between the extent of abrupt increase in turbidity by bleaching and the $CaCl_2$ or $MgCl_2$ concentration. The maximum increase

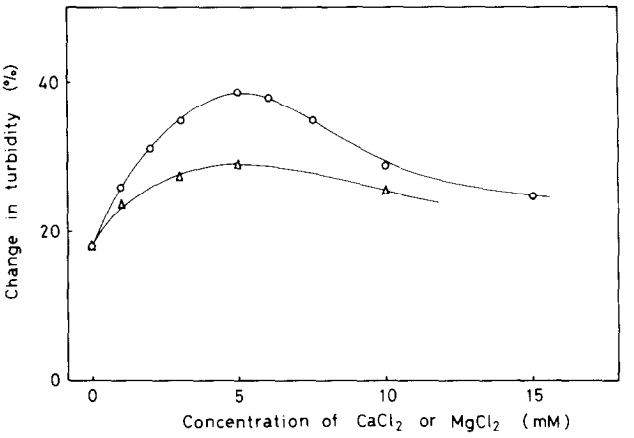

Fig. 3: The relationship between the increase in turbidity of ROS suspensions at 700 nm by bleaching and $CaCl_2$ or $MgCl_2$ concentration. Conditions; 100 mM sucrose and 0.1 % (V/V) acetic acid (pH 5.0). o, $CaCl_2$; Δ, $MgCl_2$.

in turbidity by bleaching was about 40 % at 5 mM $CaCl_2$, and about 28 % at 5 mM $MgCl_2$. The increase in turbidity by bleaching was about 6.9 % in the presence of 1 mM EDTA. In short, the maximum effect appeared at 5 mM $CaCl_2$ or $MgCl_2$, and the percent increase in the $CaCl_2$-containing suspension was greater than that in the $MgCl_2$-containing suspension over the range from 0 to 10 mM. Thus, the turbidity of an ROS suspension increased rapidly on rhodopsin bleaching. Such a rapid increase in turbidity is remarkable in the presence of divalent cations, particularly Ca^{2+}. It indicates that rhodopsin bleaching caused a change in the

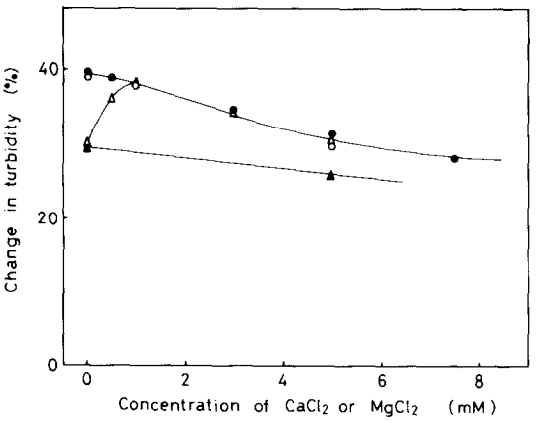

Fig. 4: Turbidity increase of ROS suspensions by bleaching in the presence of $CaCl_2$ and $MgCl_2$. Conditions; 100 mM sucrose and 0.1 % (V/V) acetic acid (pH 5.0). o, $CaCl_2$ + 5 mM $CaCl_2$; ●, $MgCl_2$ + 5 mM $CaCl_2$; Δ, $CaCl_2$ + 5 mM $MgCl_2$; ▲, $MgCl_2$ + 5 mM $MgCl_2$. Turbidity was measured at 700 nm.

conformation of the ROS membrane, resulting in concomitant aggregation of the ROS vesicles. Thus, as a result of a light-accelerated conformational change of the membrane structures, an aggregation occurred rapidly. Furthermore, the light-accelerated turbidity change was observed to be biphasic with increasing Ca^{2+} (Mg^{2+}) concentration. It should be noted that generally the rigidity of a biological membrane increases when divalent cations are bound to the membrane. Therefore, an increase in the rigidity of the ROS membrane might be expected upon the addition of divalent cations, reducing the extent of light-accelerated turbidity increase of the membrane suspension. This effect would reach a maximum when the membrane was completely loaded with divalent cations. Thus, divalent cations have two kinds of effects on the light-accelerated turbidity change: one is an enhancement, and the other is a suppression of the turbidity increase.

As the $MgCl_2$ concentration was increased from 0 to 5 mM in the presence of 5 mM $CaCl_2$, the extent of the turbidity increase decreased linearly from 38 % to 30 %. On the other hand, as the $CaCl_2$ concentration was increased from 0 to 1 mM in the presence of 5 mM $MgCl_2$, the extent of the increase was enhanced from 31 % to 36 %. However, as more $CaCl_2$ was added, the behavior became similar to that in the presence of $CaCl_2$ alone (see Fig. 4). In other words, these results indicate that the effect of Ca^{2+} on the turbidity by bleaching is the same as that of Mg^{2+} if a minute amount of Ca^{2+}, in addition to the Mg^{2+}, is present in the solution. Thus, the turbidity increase in the presence of 5 mM Mg^{2+} was greatly enhanced by the addition of a minute amount of Ca^{2+}. This indicates that Ca^{2+} have a more specific effect on the light-accelerated turbidity increase of the ROS membrane than Mg^{2+}. It is suggested that there are two kinds of binding sites for Ca^{2+} in the ROS membrane: one is a Ca^{2+} specific site, and the other is a nonspecific site to which Mg^{2+} can also bind. It is considered that some divalent cations, probably Ca^{2+}, can bind to a specific site of the ROS membrane when the conformational change of the membrane is induced by the formation of diprotonated metarhodopsin II_{380} or NRO_{440}. It is noted here that the very small turbidity change of the membrane in the dark, shown by the dotted line of Fig. 1, is not affected by the presence or absence of divalent cations.

Finally, the diprotonation of metarhodopsin II_{380} by irradiation may cause changes in membrane permeability to ions such as N^+ or K^+, resulting in the electrical changes required for excitation.

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