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ENERGY TRANSFER IN THE RHODOPSIN VISUAL SYSTEM

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ABSTRACT

A polarization of fluorescence technique has been used to test for energy transfer in bovine retinal rod outer-segments. Studies using fragments of the outer segments in a glycerol glass at low temperatures indicate that the rhodopsin fluorescence is completely depolarized when excited by polarized light. Metarhodopsin II, a product obtained by bleaching rhodopsin, does not yield completely depolarized fluorescence.

These observations indicate that excited rhodopsin molecules efficiently transfer energy by some radiationless process whereas metarhodopsin II molecules cannot.

INTRODUCTION

A number of investigations have demonstrated that the vertebrate retinal rod can be excited by the absorption of one photon (Hecht, et al. 1942). If this is the case, then in some way each visual pigment molecule must be linked with the excitation mechanism of the cell. The great number of rhodopsin molecules in a receptor cell suggests that this linkage is only an indirect one and in at least one model of this system one may visualize a large number of rhodopsin molecules acting as light gatherers and a smaller number of other sites functioning as excitation centers. This model is of course similar to that proposed for the chlorophyll light gathering system. It requires some mechanism in the retinal rod for the transfer of the excitation energy to the active site. Some form of radiationless transfer of energy is immediately suggested.

Hagins and Jennings (1959) attempted to detect energy transfer in visual system components using polarized light. In that study it was concluded that energy transfer probably did not occur but rather the depolarization effects observed were due to in situ oscillations of the rhodopsin chromophores. For several reasons to be discussed later, this work must be considered inconclusive.

More recently, Pak and Helmrich (1968) presented evidence which suggested that some form of radiationless

transfer of energy does occur in the retina. In these experiments, polarized light was used to elicit the retinal early receptor potential (ERP). With frozen and with gluteraldehyde fixed retinas these workers showed that the direction of polarization of the stimulus light did not affect the magnitude of the ERP and that repeated flashes gave results that were completely in accord with a radiationless migration of excitation. In this study, it is assumed that molecular motion probably is not important at -10°C or in the fixed retinas.

In the present study, the polarization of the fluorescence of rhodopsin (Guzzo and Pool, 1968) was used as a test of the energy migration hypothesis. Our experiments were carried out at liquid nitrogen temperatures where little chance of molecular motion exists. Thus if we excite the rhodopsin fluorescence with polarized light we expect, under ideal conditions, that little depolarization will occur in the emission if no transfer exists. If transfer occurs to a large extent, then complete depolarization should be observed.

The experimental problem, however, is considerably more difficult because scattering depolarization effects may be dominant. Thus any observation of depolarization may be meaningless unless the technique and sample can be calibrated and the scattering minimized. In our experiments the fluorescence of metarhodopsin II was used as the standard

to see whether scattering depolarization was overwhelming. Energy transfer from metarhodopsin II is not expected to occur (Guzzo, Pool and Leman, 1969). If in a given sample this fluorescence signal was observed to be polarized and the rhodopsin fluorescence from the same sample depolarized, then this would appear to be good evidence for energy transfer.

EXPERIMENTAL

The Aminco-Bowman Spectrophotofluorometer was the basic instrument used in obtaining the fluorescence spectra presented here; however, a Bausch and Lomb high intensity monochromator and xenon light source was substituted for the Aminco light source in order to minimize scattered light. A further reduction of scattered light was obtained by use of interference filters. An IP 21 photomultiplier operated at 900 volts was the detector and the shot noise was lowered by an appropriate R-C circuit. Light polarizations were obtained using "Polaroid" filter material HN22 obtained from the Polaroid Corporation. Vertical polarization of the exciting light was used exclusively in this study and correction factors for instrumental response due to the direction of emission polarization were not applied (This appears valid since we are concerned only with a comparison between the behavior of the metarhodopsin II fluorescence and that

of rhodopsin in the same sample, i.e., we are concerned with a difference in behavior and not with absolute magnitudes of polarizations).

Isolated rod outer-segments were prepared from bovine retinas as described elsewhere (Leman, 1969). The rod fragments were centrifuged into a mass and the supernatant poured off. The actual sample was prepared by mixing a small portion of these wet rod fragments with a small quantity of glycerine. This glycerine-water mixture was layered on a sample holder as a thin film. A quartz tube was then put over the film holder and the assembly immersed in liquid nitrogen held in a quartz Dewar. This apparatus is shown in Figure 1. The film, which appeared quite clear at this temperature, was maintained at an angle of approximately 45° to the excitation beam. The fluorescence of either the metarhodopsin or rhodopsin species was produced by an excitation wavelength of 425 nm.

At liquid nitrogen temperature, illumination of rhodopsin with 425 nm light will cause only a partial conversion to prelumirhodopsin and a steady state level of rhodopsin is obtained (Yoshizawa and Wald, 1963). It was from this state that the rhodopsin fluorescence was obtained. In order to prepare metarhodopsin II the rhodopsin sample was removed from the Dewar and allowed to warm in bright white light. The color change was obvious and after about one minute at

room temperature the sample was reinserted into the Dewar, cooled, and the metarhodopsin emission was easily observed (Guzzo and Pool, 1968).

A number of experiments were also carried out using the rod fragment suspension layered as a film directly on the specimen holder, i.e., the glycerine was not included. In these cases, the optical quality of the film was much poorer and it appeared chalky or cloudy at low temperatures.

Since we were forced to use quite dilute films of rod fragments in order to reduce self-absorption effects, the fluorescence intensity observed was quite weak whereas the scattered light was quite intense. To isolate the actual contribution from rhodopsin, the curves obtained were resolved into a large scattering peak whose $\lambda_{\max}$ was set at the excitation wavelength which was used to obtain the emission curve. An example of this breakdown is given in Figure 2. Here the resolution was obtained automatically using the Dupont Model 310 curve resolver. In order to compare the vertically excited fluorescence obtained from either vertical examination (Figure 2, curve a) or horizontal examination (Figure 2, curve b), the synthesized scattering curve ($\lambda_{\max}=425$) and rhodopsin fluorescence envelope ($\lambda_{\max}=550$), assumed gaussian in shape, were added so as to duplicate one observed spectrum. Usually the vertically excited horizontal emission (curve b) was synthesized first (curve d + curve e sum to curve b). Then only the scattering

intensity was changed to see if the vertically excited vertical emission could be duplicated (curve a). At this stage no changes were made in any line positions or shapes. Thus if an increase in scattering intensity (to yield curve c) with no other changes, along with the V-H rhodopsin emission (curve e) could reproduce the total VV curve (curve a), we assumed complete depolarization in the rhodopsin fluorescence. If, in order to duplicate the VV curve, we were forced to increase the intensity of the VH rhodopsin emission, then we assumed a retention of polarization.

RESULTS AND DISCUSSION

In this investigation several early attempts to measure energy transfer, using slightly modified techniques, were found to be inconclusive. It is of some value to recount briefly these experiments and note why they failed.

Our earliest experiments utilized controlled bleaching of rhodopsin by polarized light. In this study, the fluorescence observed from a rod preparation (the rhodopsin emission) was scanned several times in succession. The polarized exciting light was intense enough that significant bleaching occurred during each scan and the intensity decrease was quite reproducible from sample to sample (see Figure 3, curves a, b, and c). The experiment involved the intensity

measurement of the first scan with vertically polarized exciting light, followed by a second scan using horizontally polarized light, and finally, a third scan, again using vertically polarized light (Figure 3, curves d, e, and f). The final intensity recorded on the third scan was always identical to the third scan intensity when no horizontal excitation was used and vertical excitation was used for all three runs.

It thus appeared that the extent of bleaching was independent of the direction of polarization and this was consistent with the depolarization expected with energy transfer. However, scattering depolarization was present to a rather unknown degree and all we could conclude was that with no transfer curve f in Figure 3 should be higher than curve c in the same figure. We concluded that it was impossible to determine whether the differences in intensity between the two curves were too small for our instrumentation to detect.

In later work we found that the scattering depolarization with these samples was quite high and we should expect, for the case of no transfer, that curve f be approximately 5% higher than curve c. This small difference could not have been reliably detected considering the very low light levels that are involved in the measurement.

In our second set of experiments, we determined the retention of polarization directly by measurement of the quantity (Albrecht, 1961)

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where I_{VV} is the rhodopsin fluorescence intensity measured using vertical excitation light and a vertical filter on the emission side. The quantity I_{VH} utilizes instead a horizontal filter on the emission side and retains vertical excitation. Both quantities were measured for the rhodopsin fluorescence which appeared superimposed upon a scattering background. The same quantities were measured in the same sample after the rhodopsin had been completely bleached. The scattering was shown to be quite polarized but the rhodopsin emission, after subtraction of the scattering background, was completely depolarized.

In this series of experiments, no glycerine was used in preparing the rod fragment films and very likely a mass of ice microcrystallites throughout the sample completely depolarized any radiation that entered the sample (Hagins, 1969). It was clear that even when glycerine is added to the rod fragment suspension some internal standard was necessary in order to calibrate our technique. For this reason, we utilized the fluorescence of the intermediate product of bleaching--metarhodopsin II. The absorption maximum (380 nm) and the emission maximum (535 nm) of this species are widely separated in energy and we do not expect energy transfer between metarhodopsin II chromophores to exist (Guzzo, Pool, and Leman, 1969). Clearly, if our measurements could show a retention of polarization in the fluorescence of metarhodopsin II and complete de-

polarization in the rhodopsin fluorescence, then this should constitute evidence in favor of transfer.

This approach, which we believe has demonstrated transfer, utilized films prepared from wet rod outer-segments suspended in a small quantity of glycerine. Scattering is significantly reduced in these samples and the metarhodopsin II fluorescence showed a retention of polarization near 20% as is illustrated in Figure 4. It is apparent that even in the glass scattering is still large.

Figure 4 shows the average of ten scans of the metarhodopsin emission obtained from a sample which had first yielded the rhodopsin fluorescence curves shown in Figure 3. Curve a in Figure 4 is for vertical excitation and horizontal readout. Curve b is for vertical excitation and vertical readout.

There appears to be a real difference between the behavior of the metarhodopsin II fluorescence of Figure 4 and the rhodopsin fluorescence which is shown in Figure 3. After synthesizing curve a with the curve resolver by adding curve d to curve e, it was impossible to reproduce curve b by merely increasing the scattering curve to c. However, if the rhodopsin fluorescence curve f is added to curve c, curve b is synthesized.

Thus the vertically excited vertical rhodopsin fluorescence is more intense than the vertically excited horizontal rhodopsin fluorescence. This shows that there is a retention of polarization in the metarhodopsin II sample that did not appear in the rhodopsin fluorescence.

At this point it is useful to examine the study of Hagins and Jennings(1959). Their work can be divided into two parts;

- 1) Their calculation of the "critical" transfer distance using the Förster equation for allowed singlet-singlet transfer(Förster, 1951) did not use the correct emission spectrum of rhodopsin nor its correct quantum yield of fluorescence. However, using the correct quantities does not give any better result since the increased overlap integral is offset by the decreased quantum yield (Guzzo, Leman, and Pool, 1969). We believe that the Förster equation does not apply to these flexible polyene systems.
- 2) In their photographic study of the fluorescence of a single rod, the emission produced was very likely not from rhodopsin and as discussed previously(Guzzo, Leman, and Pool, 1969) energy transfer should not be expected.

In our measurements presented here, we use the emission of rhodopsin and standardize the depolarization measurement internally. Since we do find a difference between the rhodopsin and metarhodopsin II emissions with regard to the fluorescence depolarization measurement we believe this does reflect the phenomenon of energy transfer.

Acknowledgements

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List of Figures

Figure 1. Quartz Dewar and Sample Holder Assembly.

The quartz tube surrounding the sample holder prevented nitrogen bubbles from forming directly on the film.

Figure 2. Rhodopsin Fluorescence Curves. a) Emission recorded using vertical excitation and vertical analysis; b) Emission recorded using vertical excitation and horizontal analysis; c) Scattering curve for vertical-vertical examination; d) Scattering curve for vertical-horizontal examination; e) Resolved Rhodopsin Emission.

Figure 3. Bleaching Studies of Polarized Rhodopsin Fluorescence Curves. In I are shown three consecutive scans of the rhodopsin fluorescence, all produced with vertical excitation. In II are shown three consecutive scans of the rhodopsin fluorescence with the middle scan having horizontal excitation and the first and third having vertical excitation.

Figure 4. Metarhodopsin II fluorescence Curves.

a) Emission recorded with vertical excitation and vertical analysis; b) Emission recorded with vertical excitation and horizontal analysis; c) Scattering for

List of Figures(continued)

vertical-vertical examination;d) Scattering for vertical-horizontal examination; e) Resolved metarhodopsin II emission using vertical-vertical examination; f) Resolved metarhodopsin II emission using vertical-horizontal examination.

Figure 1

Fig. 2

Fig. 3

Fig. 4.