THE FLUORESCENCE FROM THE CHROMOPHORE OF THE PURPLE MEMBRANE PROTEIN

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ABSTRACT The fluorescence from the purple membrane protein (PM) of *Halobacterium halobium* and its relation to the primary photochemical events have been studied. The emission spectrum at 77°K has structure, with peaks at 680, 710–715, and 730–735 nm. The excitation spectrum shows a single peak centered at 580 nm. This and a comparison of the fluorescence intensity at 77°K under a variety of conditions with the amounts of the bathoproduct (or K, the only photoproduct seen at this temperature) formed suggest that the source of the fluorescence is the purple membrane itself, not the photoproduct. From the difference in several of their properties, we suggest that the fluorescing state of the pigment is different from the excited state which leads to photoconversion.

Light energy absorbed by the purple membrane (PM) of *Halobacterium halobium* (bacteriorhodopsin) can be converted into chemical free energy of a proton gradient across the cell membrane (Oesterhelt and Stoeckenius, 1973). This pumping of protons across the membrane is associated with the formation and decay of a series of spectroscopic intermediates, the first of which is formed photochemically. The following is a schematic representation of the cyclic reaction of PM568 (Lozier et al., 1975):

$$\text{PM568} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{K628} \rightarrow \text{L} \rightarrow \text{M412} \rightarrow \text{N} \rightarrow \text{O}$$

In the primary photochemical event, $\text{PM} \xrightarrow{h\nu} \text{K}$, the photon's energy is used to convert PM into a high free-energy species, K, whose free energy is used to drive the rest of the cycle (Rosenfeld et al., 1977).

In photosynthesis, where the light-to-chemical free energy-transducing system has been studied in the greatest detail, much information has been obtained by studying a small fraction of the light that is not utilized photochemically but is reemitted as fluorescence. Recently, we (Ebrey et al., 1976; Alfano et al., 1976) and others (Lewis et al., 1976; Sineshchekov and Litvin, 1976; and Gillbro et al., 1977) have studied the fluorescence from the purple membrane of *H. halobium*. In this paper, the fluorescence emission and excitation spectra (at room temperature and −196°C) and the quantum

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yield (at room temperature) of purple membrane are discussed in relation to its primary photochemical events. A firm identification of the fluorescent species, the relationship of the fluorescing state to the photochemically active state, and a study of energy transfer between chromophores are given.

MATERIALS AND METHODS

The purple membrane was prepared from *H. halobium* by the method of Becher and Cassim (1975). For all experiments reported here, the purified purple membrane samples were suspended in distilled water.

The spectrofluorometer used for the measurements has been described by Shimony et al. (1967). For measurements at −196°C, a piece of cheesecloth was soaked with about 0.5 ml of the purple membrane sample (optical density λ_{max} = 1.2, for 1-cm path length). The cheesecloth was placed at the bottom of a flat, optically clear Dewar flask which could then be filled with liquid nitrogen. Fluorescence was excited from the bottom with monochromatic light (Bausch and Lomb 0.5 M monochromator [Bausch & Lomb Inc., Rochester, N.Y.] plus Baird Atomic interference filters [Baird Atomic, Inc., Bedford, Mass.]), and collected from the front surface; the fluorescence emission passed through another Bausch and Lomb 0.5 M monochromator to the photomultiplier. The signal was amplified through a Tektronix 502 oscilloscope (Tektronix, Inc., Beaverton, Ore.) and recorded on an Esterline Angus recorder (Esterline Angus Instrument Corp., Indianapolis, Ind.) having a time constant of 0.1 s. Appropriate glass cutoff filters were used to eliminate the scattered light. Effects due to reabsorption of fluorescence were negligible because of front surface illumination and measurement and because of the very small overlap between absorption and fluorescence emission.

The purple membrane protein exists in two spectroscopically different forms—the dark- (PM558) and light-adapted (PM568) forms. When the dark-adapted form is illuminated, the absorption maximum shifts from 558 to 568 nm. All experiments were done with the light-adapted form. For the −196°C experiments, the light-adapted sample was prepared by exposing the purple membrane suspension to white light at room temperature and then waiting in the dark for approximately 1 min to allow the intermediates to decay before being cooled to −196°C. For room temperature measurements, 1 ml of the sample was placed in the bottom of the Dewar flask without the cheesecloth.

Absorption spectra at −196°C were measured using a Cary 118C spectrophotometer (Cary Instruments, Fairfield, N.J.) and a glass Dewar flask with flat windows.

The relative quantum yield of fluorescence was obtained by comparison with that of chlorophyll *a* in 80% acetone; the yield for chlorophyll was assumed to be 0.3 (Weber and Teale, 1957; Das and Govindjee, 1975).

RESULTS

Only a very small fraction of the light absorbed by PM568 is remitted as fluorescence, and it is therefore necessary that we carefully establish what species might be present during an experiment in addition to the pigment, and the possible contribution of these species to the fluorescence.

In the light-induced cyclic chain of reactions of PM568, only the first reaction, PM568 $\xrightarrow{h\nu}$ K628, is photochemical. K is stable at −196°C; upon warming in the dark, it goes through the intermediates L and M, and finally cycles back to PM. Irradiation of K at −196°C with long wavelength light, e.g., 640 nm (or longer), photo-

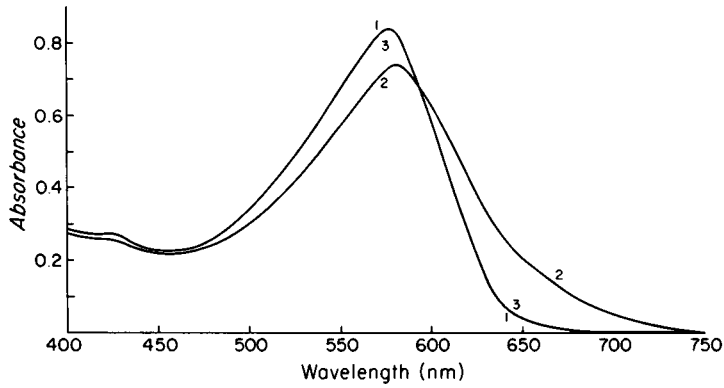

FIGURE 1 Absorption spectrum of purple membrane from *H. halobium* in 66% glycerol at −196°C. Curve 1, light-adapted; curve 2, after preillumination at −196°C with 560 nm light; curve 3, after further preillumination with 640 nm light.

chemically converts it back to PM (Lozier et al., 1975). The absorption maximum of PM at −196°C (see Fig. 1, curve 1) is at 578 nm. Upon illumination, PM is partly converted into the K form (Fig. 1, curve 2); this corresponds to the absorption spectrum of PM plus K. Further irradiation of this sample with 640-nm light, absorbed primarily by K, drives this species completely back to PM (Fig. 1, curve 3). Although slight discrepancies exist (Hurley and Ebrey, 1978), the near perfect isosbestic point seen in the

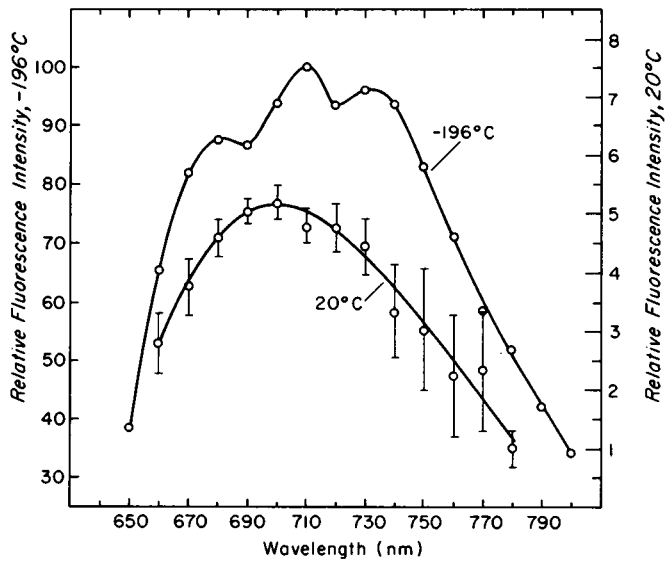

FIGURE 2 Corrected fluorescence emission spectra of light-adapted purple membrane at −196°C and 20°C. λ excitation, 580 nm (optical bandwidth, 9.9 nm); 580-nm interference filter in the exciting beam; λ measuring varied (optical bandwidth, 6.6 nm); CS 2-61 glass cutoff filter on the analyzing monochromator.

absorption spectra of PM to K conversion implies that only two species are present at −196°C: PM and K.

Emission Spectra

The corrected fluorescence emission spectra of PM at 77°K and 20°C are shown in Fig. 2. The emission spectrum at 77°K has three distinct peaks at 680, 710, and 730–735 nm. The emission spectrum (Fig. 2) is very broad and exhibits a very large (>3,000 cm^{-1}) Stokes's shift; i.e., there is a very small overlap of emission spectrum with the absorption spectrum. Because the absorption spectrum shows a single peak even at −196°C, the structure in the emission spectrum, presumably due to emissions from different vibrational bands of PM, is somewhat unexpected. The fluorescence intensity at 20°C is 15- to 20-fold lower than at −196°C, and the emission band is very broad.

Identification of the Fluorescing Species

We will be primarily concerned with the fluorescence measurements at −196°C for two main reasons. First, the quantum yield of fluorescence at −196°C is 15–20 times greater than at room temperature. Second, at −196°C only two species are present, PM and K. Thus, these are the only possible pigment-associated contributors to the emission spectra at this temperature.

One other possible source of fluorescence that must be briefly considered is impurities. The only impurity we have spectrally observed are the carotenoids, which absorb maximally at ca. 550 nm and are found primarily in the nonpurple membrane part of the bacterial plasma membrane invariably contaminating all purple membrane preparations. However, we have found that when a fraction obtained during the puri-

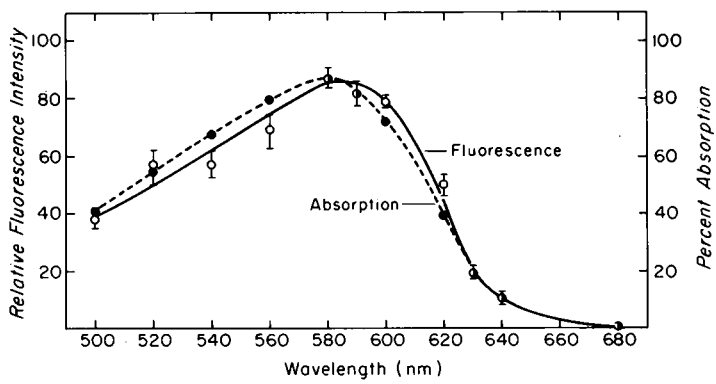

FIGURE 3 A: Corrected fluorescence excitation spectrum of light-adapted purple membrane at −196°C. Excitation optical bandwidth, 6.6 nm; λ measuring, 720 nm; optical bandwidth, 9.9 nm; two CS 2-64 glass cutoff filters before the analyzing monochromator. Sample preilluminated with λ >640 nm before each excitation. B: Percent absorption of PM. It is not possible to record the actual absorption spectrum of our sample *in situ*, so we have estimated its absorption by taking the absorption spectrum of purple membrane at 77°K and assuming an optical density of 0.5.

fication procedure of the purple membrane, consisting almost entirely of carotenoids, is separately examined, no fluorescence can be detected. Moreover, we have also examined several "carotenoidless" mutants that have much smaller amounts of carotenoids than the wild-type; these mutants appear to have a fluorescence emission spectrum and yield identical to the wild-type (see also Lewis et al., 1976).

The primary evidence that the species responsible for the fluorescence is PM comes from the excitation spectrum at −196°C. Fig. 3 shows the corrected fluorescence excitation spectrum (for emission at 720 nm). Even at −196°C, the yield is so low that the exciting light has to be quite intense; as a consequence, even within a short period of time, every pigment molecule present absorbs light and has a chance of being converted to the bathoproduct K. Because the fluoresence intensity decreases with time during the photoconversion of PM to K (see below), all excitation spectrum data points indicate the *initial* fluorescence intensity after preillumination with light of $\lambda > 640$ nm. (Preillumination with long wavelength light drives all pigment to the PM form before excitation.) The excitation spectrum has the same λ_{max} as the absorption spectrum at −196°C but is slightly broader, perhaps due to the high optical density of the sample. Although it is not possible to determine the absorption of the sample absorbed on the cheesecloth, the percent absorption curve for a sample of PM having an optical density of 0.5 is shown for comparison in Fig. 3. The similarity of the excita-

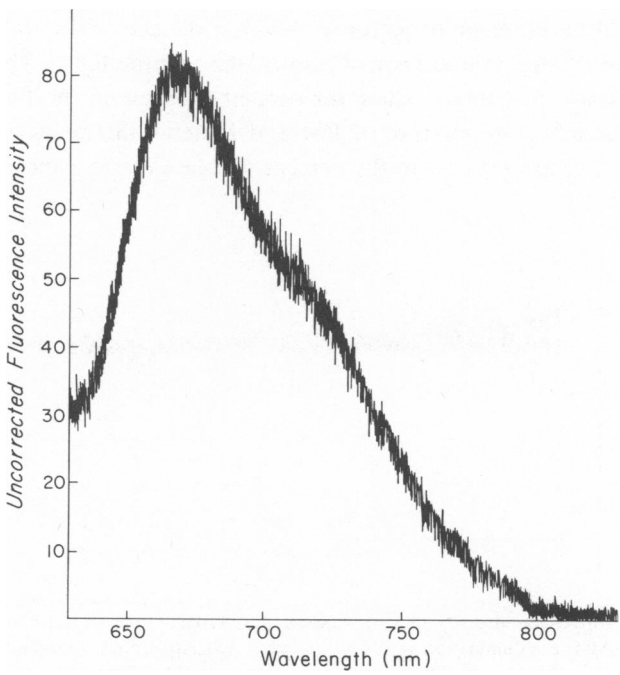

FIGURE 4 Uncorrected fluorescence emission spectra of light-adapted purple membrane at −196°C normalized at 670 nm. λ excitation, 540, 580, or 600 nm. Conditions are the same as in Fig. 2.

tion spectrum with the percent absorption spectrum of PM indicates that the fluorescence emission results only from excitation of PM. However, from this experiment alone, it cannot be determined if K itself fluoresces.

Is K Fluorescent?

To establish whether K is fluorescent, we have studied (*a*) emission spectra of samples containing different PM:K ratios; (*b*) the time-course of fluorescence after preillumination with light of $\lambda > 590$ nm; and (*c*) fluorescence emission on excitation of K alone in a sample containing a maximum amount of K.

(*a*) The emission spectra of purple membrane are identical for several excitation wavelengths (540, 580, and 600 nm). Fig. 4 shows the uncorrected emission spectra, normalized at 670 nm, obtained with 540, 580, and 600 nm excitation. In each case, the sample was preilluminated with 640 nm light in order to convert the sample into the PM state. In their photosteady states, these three exciting wavelengths should produce different ratios of PM:K (Hurley and Ebrey, 1978). Thus, even though the ratio of PM:K is different in each case, the normalized emission spectra are identical. This experiment indicates either that K is nonfluorescent or that its emission spectrum is identical with that of PM (see also Lewis et al., 1976).

(*b*) When a sample initially in the PM state (preilluminated with 640 nm light) is excited at the isosbestic point by 590 nm light, the fluorescence intensity at 680 nm decreases rapidly with time until it reaches a steady level after 8–10 s (see Fig. 5 A). We have interpreted this result to be correlated with the conversion of fluorescent PM to nonfluorescent K due to photoconversion by the exciting light. This correlation is shown quantitatively in Table I, where the percent decrease in the fluorescence upon formation of a steady-state mixture of PM and K at an illuminating wavelength is, within experimental error, equal to the percent decrease in the concentration of PM.

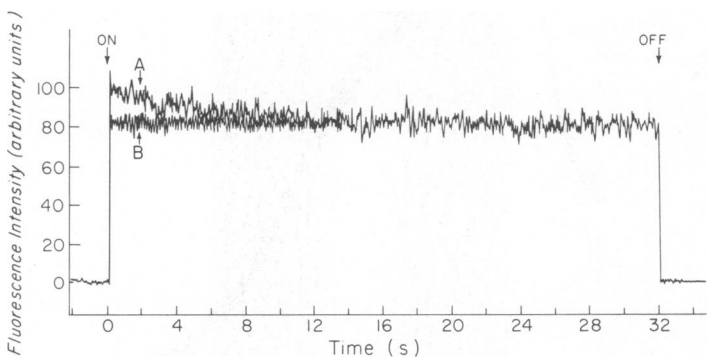

FIGURE 5 Fluorescence intensity of light-adapted purple membrane as a function of time at −196°C. (A) After preillumination with 640 nm light. (B) After preillumination with 590 nm light. Measuring optical bandwidth, 9.9 nm; excitation optical bandwidth, 6.6 nm; λ excitation, 590 nm with 590-nm interference filter in the exciting beam; λ measuring, 680 nm with CS 2-61 filter before the analyzing monochromator.

TABLE I

FLUORESCENCE MEASURED AT −196°C*

λ Excitation	Percent K628 in steady state with λ‡	Percent decrease in fluorescence at 720 nm
nm		
500	0.28	0.24 ± 0.04
520	0.28	0.23 ± 0.06
540	0.27	0.24 ± 0.04
560	0.24	0.23 ± 0.05
580	0.22	0.23 ± 0.04
585	0.20	—
590	—	0.17 ± 0.04
595	0.17	—
600	0.15	0.16 ± 0.03
606	0.13	—
620	—	0.09 ± 0.05
625	0.065	—
630	—	No change
640	—	No change

*Sample was preilluminated with 640 nm light before each measurement.
‡Hurley and Ebrey, 1978.

This implies that PM does fluoresce and K does not fluoresce. (See, however, the Discussion section for further consideration of this result.)

The relation between the decrease in fluorescence intensity with time and the conversion of PM to K is also demonstrated by preilluminating the sample with 590 nm light (producing a photostationary-state concentration of K before excitation); in this case, the fluorescence intensity does not change with time (see Fig. 5 B), and is equal to the steady-state level of Fig. 5 A. By comparison, if the sample is preilluminated with 540 nm light, then a slight increase in fluorescence is observed with 600 or 610 nm exciting light due to the conversion of some of the K to PM.

(*c*) Finally, if a photosteady state containing ca. 28% K is formed by irradiation with 500 nm light (Hurley and Ebrey, 1978) and the sample is then irradiated at 680 nm where K absorbs light and PM does not, no fluorescence can be detected. This indicates that if K alone is excited, there is no fluorescence.

Therefore, these results indicate that the fluorescence seen in our preparations of purple membrane protein at −196°C arises from the purple membrane pigment itself, not from any contaminants present nor from the primary photoproduct, K, rapidly formed at −196°C by the exciting light.

Quantum Yield of Fluorescence

The relative quantum yield of fluorescence of the purple membrane is extremely low at room temperature. Assuming the quantum yield of chlorophyll *a* fluorescence in 80% acetone to be 0.30 (Weber and Teale, 1957; Das and Govindjee, 1975), the quantum

yield of fluorescence of purple membrane was determined to be approximately 2.4×10^{-5} by comparison of the integrated areas under the emission curves. The intrinsic lifetime, τ_0, as calculated from the main absorption band, is approximately 6 ns. Thus, the lifetime (τ) should be approximately 1.4×10^{-13} s. The lifetime (τ) inferred from the measurement is much longer, 3×10^{-12} s (Alfano et al., 1976). Hirsch et al. (1976) have found an even longer lifetime—15 ps. This discrepancy between the measured and calculated lifetimes suggests that the fluorescence may originate from a forbidden state, as suggested by Hudson and Kohler (1972) for other polyenes (see also Alfano et al., 1976). At −196°C the fluorescence lifetime is ca. 40 ps (Alfano et al., 1976), considerably longer than that seen by Kaufmann et al. (1976, and private communication) for the time of formation of the K ground state after PM is excited.

DISCUSSION

We have shown that the species giving rise to the low yield fluorescence observed from suspensions of purple membrane at −196°C is the pigment itself, PM568, not a contaminant or the photoproduct formed by the exciting light. The excitation spectrum, the decrease in fluorescence upon excitation of a PM568 sample, and the emission spectra of various photosteady mixtures of PM and K all suggest that all the fluorescence observed can be assigned to PM568. Lewis et al. (1976) have also reported fluorescence from a purple membrane suspension at −196°C, but their emission spectrum is considerably different from that which we have shown here to be due to PM. At present, the relationship between these two emissions is unclear.

Energy Transfer

Energy transfer has been shown to take place in purple membrane from PM to K (Hurley and Ebrey, 1978). Two questions present themselves concerning energy transfer in purple membrane. First, is there energy transfer from K to PM? We cannot yet answer this question but we can answer a more restrictive one—is there energy transfer from K to the fluorescent state of PM? The third experiment of the previous section shows clearly that light energy absorbed by K cannot be transferred to an energy level of PM from which fluorescence can take place. If it were, we should observe PM fluorescence when we selectively irradiate K. One interpretation of this result is that thermalization is extremely rapid so that energy transfer takes place from a partially thermalized excited state. Then, because the energy gap between the thermalized excited state and the ground state of K is much smaller than the gap to the ground state of PM, there would not be enough energy in the partially thermalized K excited state to excite PM by energy transfer.

The second question is what does the fluorescence yield in the photosteady state (containing PM and K) imply about energy transfer from PM to K? Hurley and Ebrey (1978) have shown that such a transfer takes place with an efficiency of ca. 45%. This should result in a percent decrease in fluorescence that is greater than the percent K formed in the photosteady state if energy absorbed by PM were transferred to the nonfluorescent K.

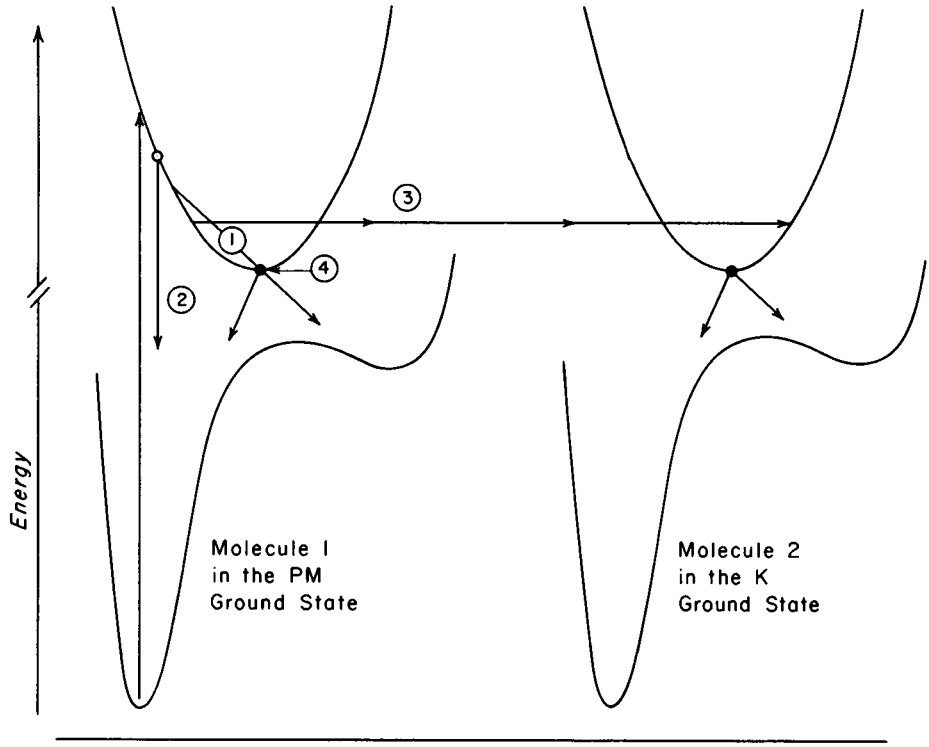

FIGURE 6 Schematic representation of the possible de-excitation pathways for light energy absorbed by a PM pigment molecule in the presence of a K pigment molecule. Starting from the Franck-Condon level involved in the absorption process, the energy may either (*1*) relax to the thermalized state; (*2*) go to the fluorescing state, which appears to be different from the state responsible for the absorption and the state from which the photochemistry takes place; (*3*) transfer to a second pigment molecule after the excitation process; or (*4*) photochemically relax to the K ground state (ϕ = 0.3) or to the PM ground state (ϕ = 0.7). This occurs after thermalization to an excited state common to both PM and K forms of single pigment molecule.

In contrast, the data in Table I show that the decrease in fluorescence is equal to the amount of K formed. That is, K does not seem to be able to quench the PM fluorescence. A simple explanation for this is that the fluorescence originates from a different state which precedes the one from which energy transfer takes place, and there is no competition between fluorescence and energy transfer. That is, we assume that energy transfer takes place from a state of PM that is not a precursor of the fluorescent state. This situation is shown in Fig. 6, and evidence for it is discussed below.

Is the Fluorescing State Identical with the Excited Photoconversion State of PM?

Several lines of evidence suggest that the excited state of PM responsible for the fluorescence is distinct from the thermalized PM excited state postulated to be common with the thermalized K excited state (Rosenfeld et al., 1977; Hurley and Ebrey,

1978). It is from this latter state that the photoconversion, PM → K and the reverse photoreaction, K → PM are believed to take place.

The first argument relies on the hypothesis that the photochemical transformations of PM and K do take place from a common thermalized excited state (Rosenfeld et al., 1976; Becher and Ebrey, 1977; Goldschmidt et al., 1977). If the fluorescence from PM were from this common state, and because this state could be populated by light absorbed by K, there should be fluorescence from K. Because there is not, this suggests that the photoconversion state is different from the fluorescing state.

Strong arguments not based on the common excited-state hypothesis can also be made: Lewis et al. (1976) and Alfano et al. (1976) have shown that the yield of fluorescence is strongly temperature dependent. We (Hurley and Ebrey, 1978) have shown that the yields of both the photochemical reactions, PM $\underset{h\nu}{\overset{h\nu}{\rightleftharpoons}}$ K, are temperature independent from 20°C to 77°K. This also suggests that the photoconversion state is different from the fluorescing state. Finally, Alfano et al. (1976) have shown that at −196°C, the lifetime of the fluorescing state is ca. 40 ps, whereas Kaufmann et al. (1976) have found that the lifetime of the state leading to K is < 10 ps. These three differences in the properties of the fluorescing state and the photochemical state suggest that they are distinct entities.

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