PICOSECOND KINETICS OF THE FLUORESCENCE FROM THE CHROMOPHORE OF THE PURPLE MEMBRANE PROTEIN OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT The fluorescence emission kinetics at 740 nm of the retinylidene chromophore of the purple membrane protein of *Halobacterium halobium* have been studied. Using picosecond laser pulses and an optical Kerr gate, the fluorescence risetime is found to be less than 8 ps and its lifetime is 40 ± 5 ps at 90°K and is estimated to be less than 3 ps at room temperature.

With the recent development of picosecond laser pulses, the primary energy conversion processes in biological pigment systems can be studied on a picosecond time scale (Busch et al., 1972; Siebert et al., 1973; Yu et al., 1975). An ultrafast optical Kerr gate (Duguay and Hansen, 1969; Shimizu and Stoicheff, 1969) operated by a mode-locked Nd:glass laser has permitted measurement of the fluorescence kinetics in photosynthetic materials (Yu et al., 1975). In this paper, we use this technique to study the fate of light energy absorbed by the chromophore of the purple membrane protein of *Halobacterium halobium.* We report the first direct measurement of the fluorescence lifetime of the emission from the purple membrane protein. The lifetime was found to be 40 ± 5 ps at 90 K and estimated to be 3 ps at room temperature.

The purple membrane of *H. halobium* contains a single protein, often referred to as bacteriorhodopsin, to which retinal is bound via a protonated Schiff base. Therefore, the bacterial pigment resembles the visual pigment rhodopsin spectroscopically (Lozier et al., 1975) and perhaps photochemically (T. Rosenfeld, B. Honig, M. Ottolenghi, and T. Ebrey, manuscript in preparation). Moreover, the purple membrane protein has been shown to utilize light energy to move protons across the cell membrane (Oesterhelt and Stoeckenius, 1973), and in this way it resembles photosynthetic systems. However, this protein seems to be an especially simple proton pumping system in that it is the only protein involved between light absorption and proton translocation. The observation of the fluorescence from the purple membrane protein and the characterization of its kinetics are of importance for understanding the electronic structures and excited states not only of this pigment but also of rhodopsin.

Recently, fluorescence has been observed from the chromophore of the purple membrane protein (R. Govindjee, B. Becher, and T. Ebrey, manuscript in preparation; Ebrey et al., 1976; Lewis et al., 1976). At room temperature, we find a broad emission centered at about 710 nm, with a yield of about 2×10^{-5}; upon cooling, the yield rises about 15-fold at 77°K. At 77°K the fluorescence consists of three fairly distinct bands of approximately the same intensity at 680 nm, 710 nm, and 740 nm (Govindjee et al., manuscript in preparation). The primary photoproduct formed upon irradiating the purple membrane protein at liquid nitrogen temperatures is similar to the bathointermediate of rhodopsin formed at these temperatures and is usually called the batho (or "K") intermediate of the bacterial pigment.

EXPERIMENTAL METHODS

The experimental apparatus to measure the fluorescence kinetics has been discussed in detail elsewhere (Yu et al., 1975). It consists of a mode-locked Nd:glass laser, a potassium dihydrogen phosphate crystal for the generation of the second harmonic, a $\frac{1}{2}$ cm sample cuvette, and a CS_2 cell optical Kerr gate. Two laser beams of picosecond duration are used in the experiment—a 0.53 μm beam with a 6 ps pulse width and a 1.06 μm beam 8 ps wide. The 0.53 μm beam (photon flux 6×10^{14} photon/cm^2 per excitation pulse, defocused to 3 mm diameter) activates the fluorescence in the sample cuvette. Approximately 100 laser pulses each separated by 7 ns were used to excite the sample on each laser shot. The 1.06 μm beam times the gate by inducing a short-lived birefringence in the CS_2 cell which is situated between crossed polarizers in the fluorescence path. The fluorescence can pass through the crossed polaroid configuration only during the short time of the induced birefringence in the CS_2 cell. The fluorescence is thus sampled at different times simply by adjusting the pathlength of the 1.06 μm beam with respect to the 0.53 μm beam. The zero point is defined at the coincidence in time and space of the 0.53 μm and the 1.06 μm beams at the CS_2 cell with water substituted for the sample. The total fluorescence intensity, the fluorescent intensity at 740 nm at a particular time delay, and the intensities of the 0.53 μm and 1.06 μm beams were detected and displayed on a four-channel analog to digital electronic integrator. A water blank had no detectable signal.

The purple membrane samples were prepared by the method of Becher and Cassim (1975). The membrane was suspended in water or water-glycerol (1:2). The optical density of the sample at 0.53 μm was adjusted to 1.5. The chromophore concentration was about 3×10^{-5} M. The light adapted form of the purple membrane (λ_{max} = 568 nm), prepared by briefly exposing the sample with light (Lozier et al., 1975) was employed in these measurements.

Because our experiment uses a train of about 100 8-ps wide pulses, each separated by 7 ns, the possibility should be considered that the early pulses in the train might produce a new species which, when illuminated by later pulses in the train, is responsible for the fluorescence we observe. This does not occur for the following reason. After illumination at liquid nitrogen temperatures, only two species are present—the original pigment and the bathointermediate, the latter having under all conditions a lower con-

centration (Govindjee et al., manuscript in preparation). Any fluorescence must be from one of these two species. In a separate experiment (Govindjee et al., manuscript in preparation) we have determined that the fluorescence yield from the bathointermediate is small, at least three times lower than that of the purple membrane. Thus, the fluorescence we observe at liquid nitrogen temperatures is from the light adapted form of the pigment itself. At room temperature, other means had to be employed to determine the fluorescence lifetime since we could obtain only an upper limit for the lifetime by direct measurement. However, three different criteria suggest that the time unresolved fluorescence we did measure was due to the pigment. First, at room temperature, the lifetime we observe (less than 8 ps; see below) is consistent with that calculated directly for the pigment at this temperature from other measurements (3 ps; see below). Secondly, for several microseconds after excitation at room temperature only the pigment itself and the low fluorescent bathointermediate are present (Kung et al., 1975). Since the length of the total excitation train is less than 0.7 μs, it is unlikely that early picosecond pulses in the train could excite any fluorescent species other than the pigment. Finally, direct measurements of the fluorescence intensity obtained from each pulse, monitored during the entire duration of the train, gave no indication of a rise in yield towards the end of the train, again suggesting that the fluorescence is not from a photoproduct created by the early pulses and excited by the later ones.

RESULTS AND DISCUSSION

The relative fluorescent intensity of purple membrane at 740 nm is plotted as a function of time in Fig. 1. Measurements were made at 90°K (Fig. 1 *a*) and at room temperature (Fig. 1 *b*). The same scale is used for relative fluorescence in the figures. The following salient points are displayed in the figure. The initial peak at both temperatures occurs very close to time zero which indicates that the risetime of the fluorescence in the sample is extremely rapid—less than 8 ps. The solid curve represents the decay envelope of the fluorescent state. At 90°K the lifetime of the fluorescence is 40 $\pm$ 5 ps (Fig. 1 *a*). At room temperature, a decay time of 8 ps is obtained for the envelope, which is approximately identical to the gate characteristic; therefore, this is the upper limit of the fluorescent lifetime. An estimate of lifetime at room temperature (300°K) was obtained from the ratio of the peak fluorescence intensity within the Kerr gate width of 10 ps at $t = 0$ for 90°K and 300°K samples. The ratio was 3, which suggests a lifetime of about 3 ps. This estimate is consistent with the ratio of the total fluorescence intensity at 90°K and 300°K of about 15, since $\tau_{300°K} = \tau_{90°K}\phi_{300°K}/\phi_{90°K} \simeq 2.6$ ps. Deconvolution from the prompt decay curve of CS_2 gate is not warranted here since the statistical variation of the data is rather large.

We calculated from the area of the 568 nm absorption band that the intrinsic radiative lifetime of this transition, τ_o, is about 6 ns, and have measured the quantum yield of fluorescence ϕ to be $\sim 2 \times 10^{-5}$ at room temperature. From our measurements of τ and ϕ and the relation $\tau = \phi\tau_o$, the intrinsic radiative lifetime, τ_0, can be calculated to be about 150 ns. This is 25 times larger than the value obtained from

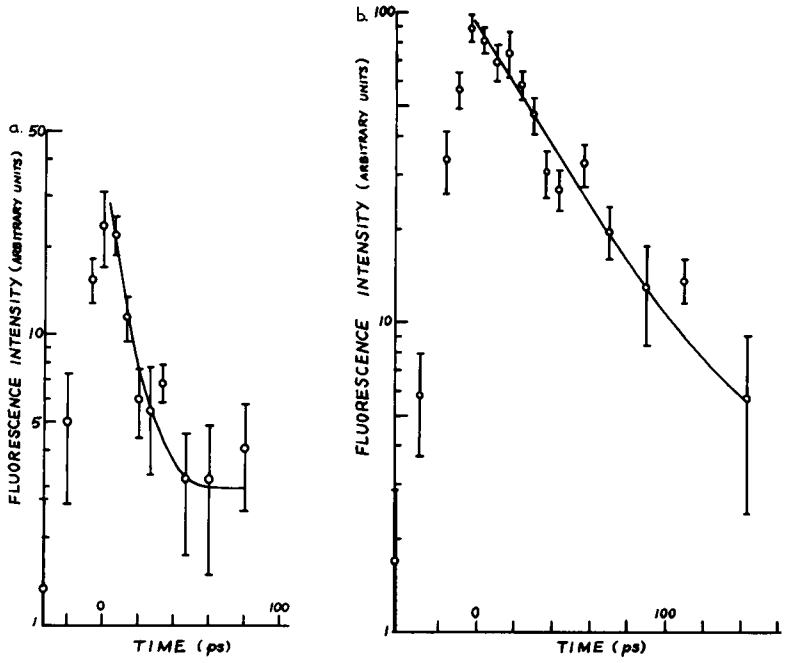

FIGURE 1 *a* Fluorescence intensity of the purple membrane protein at 740 nm and 90°K vs. time. The error bar is the standard deviation of the mean of six or more laser shots. The solid curve is $I = A \exp(-t/\tau) + B$, where $A = 95$, B (noise background) = 3, and $\tau = 40$ ps.
FIGURE 1 *b* Fluorescence intensity of the purple membrane protein at 740 nm and room temperature vs. time. The error bar is the standard deviation of the mean of six or more laser shots. The solid curve is $I = A \exp(-t/\tau) + B$, where $A = 35$, B (noise background) = 3, and $\tau = 9$ ps.

absorption spectrum. A similar discrepancy has been observed in other polyenes (Hudson and Kohler, 1974). The long radiative lifetime of the purple membrane (150 ns) may indicate that the emission is from a forbidden state buried under the 568 nm band, perhaps the $^1A_g^-$, which has been proposed to exist in many polyenes (Hudson and Kohler, 1974). Neporent (1973) has suggested an alternative explanation of the seemingly anomolous radiative lifetimes of polyenes in terms of his four-level scheme.

The observed fluorescence lifetime is quite unusual because it is so short, 40 ps at 90°K and extrapolated to be 3 ps at room temperature. This rapid decay of the excited state of the chromophore in the purple membrane protein must be due to a nonradiative process—the primary photochemistry, probably isomerization (Rosenfeld et al., manuscript in preparation) and/or radiationless deexcitation. Therefore, the 3 ps lifetime at room temperature may be the time constant for the appearance of the bathointermediate of the purple membrane. In the case of the visual pigment rhodopsin, a time constant of less than 6 ps has been reported for the formation of bathorhodopsin (Busch et al., 1973).

Note Added in Proof: The proteins comprising the purple membrane are organized into trimers; the three chromophores are close enough to each other to give rise to an exciton interaction (Becher and Ebrey, *Biochem. Biophys. Res. Commun.*, in press). To test whether this exciton interaction could affect the lifetime, noninteracting purple membrane protein monomers were prepared in two different ways (see Becher and Ebrey); the resulting fluorescent lifetimes of these preparations were unchanged from that of the intact membrane trimers.

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REFERENCES

BECHER, B., and J. CASSIM. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep. Biochem.* **5**:161.

BUSCH, G., M. APPLEBURY, A. LAMOLA, and P. RENTZEPIS. 1972. Formation and decay of prelumirhodopsin at room temperatures. *Proc. Natl. Acad. Sci. U.S.A.* **69**:2802.

DUGUAY, M., and J. HANSEN. 1969. The ultrafast light gate. *Appl. Phys. Lett.* **15**:192.

EBREY, T., R. GOVINDJEE, B. BECHER, R. ALFANO, and W. YU. 1976. Fluorescence from the chromophore of the purple membrane protein of *H. halobium*. *Biophys. J.* **16**(2, pt. 2):99*a*. (Abstr.).

HUDSON, B., and B. KOHLER. 1974. Linear polyene electronic structure and spectroscopy. *Ann. Rev. Phys. Chem.* **25**:437.

KUNG, M., D. DEVAULT, B. HESS, and D. OESTERHELT. 1975. Photolysis of bacterial rhodopsin. *Biophys. J.* **15**:907.

LEWIS, A., J. SPOONHOWER, and G. PERREAULT. 1976. The observation of light emission from a rhodopsin. *Nature* (*Lond.*). In press.

LOZIER, R., R. BOGOMOLNI, and W. STOECKENIUS. 1975. Bacteriorhodopsin: a light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* **15**:955.

NEPORENT, B. 1973. Intramolecular relaxation and luminescence of molecules of complex organic compounds. *Izv. Akad. Nauk SSR Ser. Fiz.* **37**:236.

OESTERHELT, D., and W. STOECKENIUS. 1973. Functions of a New Photoreceptor Membrane. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2853.

SHIMIZU, F., and B. STOICHEFF. 1969. Study of the duration and birefringence of self-trapped filaments in CS_2. *IEEE Quantum Electron.* **5**:544.

SIEBERT, M., R. ALFANO, and S. SHAPIRO. 1973. Picosecond fluorescent kinetics of in vivo chlorophyll. *Biochem. Biophys. Acta.* **292**:493.

YU, W., P. HO, R. ALFANO, and M. SIEBERT. 1975. Fluorescent kinetics of chlorophyll in photosystems I and II enriched fractions of spinach. *Biochem. Biophys. Acta.* **387**:159.