BRIEF COMMUNICATION

FLUORESCENCE RELAXATION KINETICS FROM RHODOPSIN AND ISORHODOPSIN

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ABSTRACT The fluorescence kinetics of bovine rhodopsin and isorhodopsin excited with a single picosecond laser pulse have been measured with a streak camera. The rise and the decay time of the intrinsic fluorescence emission from rhodopsin and isorhodopsin are found to be <12 ps.

INTRODUCTION

The visual pigment, rhodopsin, consists of a chromophore 11-*cis* retinal covalently bound to a protein through a protonated Schiff base. The protein binding site also accommodates a 9-*cis* retinal. This pigment, called isorhodopsin, has the same bleaching sequence as rhodopsin (Yoshizawa and Wald, 1963). Picosecond absorption techniques have been used to study the primary event in vision (Busch et al., 1972; Green et al., 1977; Peters et al., 1977; Monger et al., 1979). One of the important problems lies in understanding the nature of the interactions between the protein and the chromophore. Fluorescence measurements can complement the absorption measurements as they provide information about the excited state of the chromophore in the binding site.

Fluorescence from rhodopsin is difficult to observe because of the expected small quantum yield (Guzzo and Pool, 1968; Alfano et al., 1976) and because fluorescence from impurities in the samples can mask the intrinsic fluorescence from rhodopsin. Guzzo and Pool (1968) have observed fluorescence from rod outer segments (ROS) at steady state. In this paper, we report on the first direct fluorescence kinetics from rhodopsin and isorhodopsin excited with a single 5-ps laser pulse at room temperature. A streak camera was used for the detection of the fluorescence.

METHODS AND MATERIALS

The apparatus used in the fluorescence kinetic measurements has been previously described (Yu et al., 1977; Schiller et al., 1980). A single pulse (1054 nm) from the output of a mode-locked Nd:glass laser was selected and amplified (Lu et al., 1979) and passed through a KDP crystal where the second harmonic of the laser at 527 nm was generated. The pulse width of the 527-nm pulse measured by two-photo fluorescence was found to be ~5 ps. This pulse was used to excite the sample and calibrate the

detection apparatus (Yu et al., 1977; Schiller et al., 1980). The exciting beam was collimated to a spot size of 1.5×10^{-2} cm^2 at the sample position. The average energy density was 2×10^{16} photons/cm^2. The sample was frontally excited and the fluorescence collected and focused onto the 30-μm entrance slit of a Hamamatsu streak camera (model C-979, Hamamatsu Corp., Middlesex, NJ). A 550-nm cut-off filter (3-67 Corning Glass Works, Science Products Div., Corning, N.Y.) was placed in the path of the fluorescence to eliminate any scattered laser beam. A complete study of the frequency as well as the temporal profile of the emission spectrum is important for the understanding of the excited states of rhodopsin. Technical problems, however, associated with pulse broadening inside the monochromator (Schiller and Alfano, 1980) and the low sensitivity of the detection system precluded a complete frequency study of the emission at this time. In this report, we have used a series of cut-off filters (R-62, R-66 Hoya Optics, U.S.A.; Menlo Park, Calif.) to determine approximately the range of the emission spectrum. It was found that the range of the fluorescence emission is below 660 nm with $\sim$>75% of the intensity below 620 nm. This is in agreement with steady-state measurements of Guzzo and Pool (1968). The integrated fluorescence emission in the region 550–660 nm was detected. A 527-nm prepulse (not shown in the figures) was used to provide a reference mark on the time axis for signal averaging. The Hamamatsu streak camera was coupled to a GBC SIT video camera and Hamamatsu temporal analyzer and interfaced to a DEC MINC minicomputer. After normalization both in time and intensity for nonlinearities in the streak rate, the data were analyzed through appropriate use of curve-fitting techniques. The time resolution (FWHM) of the complete apparatus (laser-streak camera) system was measured to be $\leq$ 12 ps.

Bovine rhodopsin was prepared by the method of Papermaster and Dryer (1973). Part of the sample was solubilized in digitonin and the absorption spectrum recorded. The absorbance ratio A280:A500 was between 3 and 4. No absorption was observed at 425 nm which could be attributed to 11-cis-retinal-protein complex (Stubbs et al., 1979). Rhodopsin ROS were suspended in potassium phosphate buffer 0.067 M pH 6.6 and 0.1 M hydroxylamine. Isorhodopsin was prepared photochemically from rhodopsin by irradiating the sample at liquid nitrogen with the 568.2-nm krypton laser line (Oseroff and Callender, 1974). Part of the isorhodopsin vesicles were solubilized in digitonin and the absorption spectrum checked for complete conversion to isorhodopsin.

Vesicle suspensions were placed in a 2-mm cuvette. The concentration was estimated to be 10^{-4} M. The sample was stirred after every shot. At the end of the experiments the samples were bleached under room light and the fluorescence measurements were repeated. Bleached samples of rhodopsin and isorhodopsin show identical fluorescence decays. All measurements were performed at room temperature.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the fluorescence kinetics of rhodopsin and isorhodopsin, respectively. Both curves show a fast spike-like component which rises and decays within the resolution of the apparatus <12 ps. A slower component follows with a rise time for rhodopsin of 57 $\pm$ 6 ps (average and standard deviation of five curves) and isorhodopsin 67 $\pm$ 9 ps (average and standard deviation of nine curves). The $1/e$ time for the decay of this component is 228 + 35 and 258 $\pm$ 55 ps, respectively. The bleached sample shows only the "slow" component (Fig. 3) with a rise time of 61 $\pm$ 4 ps (four curves) and $1/e$ decay time of 343 $\pm$ 36 ps. The measurements presented here were taken at an intensity density of 2×10^{16} photons/cm^2. The "fast" component of both rhodopsin and isorhodopsin can be observed at lower energy densities down to $\sim 2 \times 10^{15}$ photons/cm^2. The intensity dependence of the fluorescence intensity of the fast component is linear within the range 2×10^{15}–2×10^{16} photons/cm^2.

The rise time and the decay kinetics of the slow component of rhodopsin, isorhodopsin, and the bleached sample are reasonably close so that this part of the kinetics is most likely caused by impurities in the sample. The fluorescence rise and decay times of the fast component are

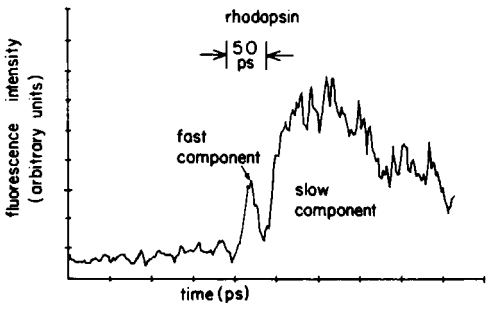

FIGURE 1

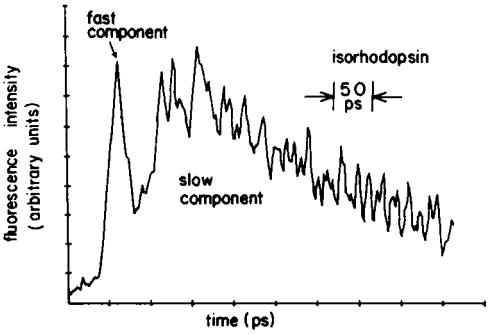

FIGURE 2

FIGURE 1 Fluorescence kinetics of rhodopsin at room temperature excited by a single 527-nm pulse for $\lambda > 550$ nm.

FIGURE 2 Fluorescence kinetics of isorhodopsin at room temperature, excited by a single 527-nm pulse for $\lambda > 550$ nm.

limited by the resolution of the streak camera being 12 ps. It is reasonable to expect that the lifetime of this component is <12 ps as has been indicated by previous experiments (Green et al., 1977) and theoretical calculations (Birge and Hubbard, 1980).

We identify the fast component with the intrinsic fluorescence from rhodopsin and isorhodopsin for the following four reasons: (*a*) The fast component appears consistently in all samples of rhodopsin and isorhodopsin. (*b*) The fast component is not present in the bleached samples. (*c*) The fast lifetime is consistent with the absorption measurements of the formation of bathorhodopsin from rhodopsin and isorhodopsin (Busch et al., 1972; Green et al., 1977) as well as at the recovery time of the ground state (Kobayashi 1980) and unpublished data. The lifetime of the fast component of the fluorescence is consistent with the molecular dynamics calculations of Birge and Hubbard (1980). Furthermore, given a quantum yield of $\phi = 0.001$ and assuming a radiative lifetime of $\tau_0 < 5 \times 10^{-9}$ s, the lifetime of the intrinsic fluorescence of rhodopsin is estimated to be $<5 \times 10^{-12}$ s. The present measurements, therefore, cannot determine whether the limiting factor in the fluorescence decay is a dynamical one due to the formation of an activated complex (Birge and Hubbard, 1980), or a statistical one associated

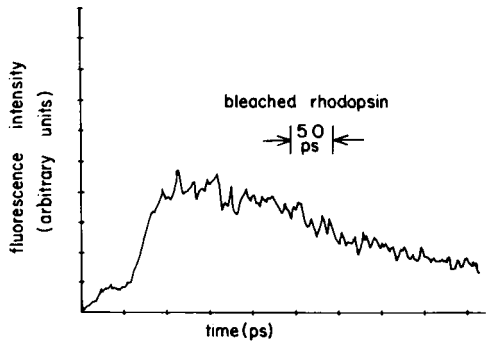

FIGURE 3 Fluorescence kinetics of bleached rhodopsin at room temperature, excited by a single 527-nm pulse for $\lambda > 550$ nm. Similar kinetics were measured in bleached isorhodopsin.

with $\phi\tau_0$. (*d*) Impurities if present, would be at low concentrations because they were not observed in the absorption spectra. Therefore, the impurities would need to have a high quantum yield to produce an effect. High quantum yield, however, implies long fluorescent lifetimes which is contrary to what we observed.

It is expected that there will be some bathorhodopsin formed during the duration of the pulse, which will be optically excited and may contribute to the fluorescence. However, as the molecular dynamics calculations show (Birge and Hubbard, 1980) the fluorescence from the activated complex of bathorhodpsin (1,500 nm) will be outside the range of detection. In any case we estimate that the amount of bathorhodopsin formed during the pulse is <20%. The fast component arises from the excited states of rhodopsin and isorhodopsin. The kinetics of the fast component are resolution limited and are consistent with the time of formation of bathorhodopsin, 3 ps (Green et al., 1977).

We thank Mr. A. Dagen, Mr. F. Pellegrino, Mr. M. Junnarkar, and Mr. R. Seymour for technical assistance, and Professor R. Callender, Dr. J. Pande, Dr. V. Baloh-Nair, and Professor Nakanishi for supplying the bovine rhodopsin and for helpful discussions.

This research is supported in part from National Institutes of Health EYO 2515 and City University of New York Faculty Research Award Program.

Received for publication 21 January 1981 and in revised form 1 April 1981.

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