Picosecond decay kinetics and quantum yield of fluorescence of the photoactive yellow protein from the halophilic purple phototrophic bacterium, *Ectothiorhodospira halophila*

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ABSTRACT The photoactive yellow protein (PYP) has been previously shown to be partially bleached and red shifted (in less than 10 ns) by a pulse of laser excitation at the wavelength maximum (445 nm), to further bleach ($k = 7.5 \times 10^3\ s^{-1}$), and then to slowly recover in the dark ($k = 2.6\ s^{-1}$) (Meyer, T. E., G. Tollin, J. H. Hazzard, and M. A. Cusanovich. 1989. *Biophys. J.* 56:559–564). The quantum yield for the formation of the fully bleached form was found to be 0.64. We have now shown that the yellow protein is weakly fluorescent with an emission maximum at 495 nm (which mirrors excitation at 445 nm) and a fluorescence quantum yield of 1.4×10^{-3}. Measurement of the picosecond kinetics of the fluorescence decay shows that ~90% of the emission occurs with a lifetime of 12 ps. This is in good agreement with the quantum yield determination, which suggests that a single quenching process (presumably the photochemical event) is primarily responsible for the excited state decay. The lifetime of the excited state of PYP is remarkably similar to that for the rise of the first photochemical intermediate of bacteriorhodopsin, and underscores the fundamental similarity in their photocycles despite a lack of structural relationship.

INTRODUCTION

The photoactive yellow protein (PYP) is a small soluble protein found in extracts of moderately to extremely halophilic purple phototrophic bacteria (Meyer, 1985; Meyer et al., 1990). PYP from *Ectothiorhodospira halophila* has a molecular weight of 14,000 and has a wavelength maximum at 445 nm (extinction coefficient $45.5\ mM^{-1}\ cm^{-1}$). The function of PYP is unknown, but it undergoes photochemical transformations (Meyer et al., 1987) which are remarkably similar to those of the sensory rhodopsins from the halophilic aerobic bacterium, *Halobacterium halobium* (Bogomolni and Spudich, 1982). The three-dimensional structure of PYP shows that the protein contains nearly all beta secondary structure, although at the present resolution the chromophore has not been identified (McRee et al., 1989). It appears to be homologous to proteins which bind hydrophobic molecules such as the cellular retinol binding protein (Newcomer et al., 1984), insecticyanin (Holden et al., 1987), fatty acid binding protein (Sacchettini et al., 1988), P2 myelin protein (Jones et al., 1988), and beta-lactoglobulin (Monaco et al., 1987). There is no structural similarity to bacteriorhodopsin which is apparently all helical (Henderson et al., 1990). After a pulse of laser light, PYP undergoes a photocycle which involves at least two intermediates (Meyer et al., 1987). A quantum yield of 0.64 for appearance of the first long-lived intermediate in the PYP photocycle (Meyer et al., 1989) is similar to that of bacteriorhodopsin (Tittor and Oesterhelt, 1990). The kinetics of formation of the completely bleached form of PYP and its recovery to the resting state (Meyer et al., 1987) are sensitive to solvent viscosity and to the presence of aliphatic alcohols (Meyer et al., 1989). The protein apparently undergoes a conformational change, which exposes a hydrophobic site to the solvent. This suggests that PYP may be designed to bind to a hydrophobic receptor when irradiated with blue light. Although the biological function of PYP is presently unknown, it has been postulated that it is the blue-light receptor for negative phototaxis in phototrophic halophiles (Meyer et al., 1989).

We have now measured the quantum yield and the picosecond kinetics of fluorescence decay to extend our observations on the PYP photocycle, and to compare it with that of bacteriorhodopsin with which it appears to be remarkably similar.

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MATERIALS AND METHODS

PYP was obtained from *E. halophila* as previously described (Meyer, 1985).

Fluorescence quantum yield measurements (Birks, 1976) were made relative to fluorescein, using a value of 0.93 for the fluorescein quantum yield (Demas et al., 1971; Magde et al., 1979). Measurements of absorption spectra of all samples were taken on a Varian Cary 219 UV/vis spectrophotometer. Corrected fluorescence emission spectra of samples of fluorescein in 0.1 M NaOH ($A = 0.0336$ at 447 nm) and photoactive yellow protein in 5 mM phosphate buffer, pH 7.0 ($A = 0.0497$ at 447 nm) were measured on a fluorolog spectrofluorometer (Spex Industries, Inc., Edison, NJ), with excitation at 447 nm. Spectral bandwidth for excitation was 1.8 nm and for emission was 5.4

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nm. A calibrated neutral density filter was used to attenuate the excitation for the fluorescein sample so that both sample and reference emission spectra could be measured accurately with identical instrumental settings. The emission spectra were converted to a wavenumber scale, integrated and the values of the integrals corrected for the slightly different sample absorbances and the attenuation by the neutral density filter for the fluorescein sample. An integral of a buffer blank was subtracted from each sample to remove a small contribution due to Raman scattering. This correction was six percent for the protein sample and negligible for the fluorescein sample. Standard methods of error propagation were applied to obtain an estimate of the uncertainty of the quantum yield, which was found to be about seven percent of the measured value. A second determination with A = 0.0272 at 447 nm gave a value just within the error quoted above.

Fluorescence lifetimes were measured by time-resolved single photon counting on an instrument described previously (Causgrove et al., 1990), except that the model 3500 dye laser (Spectra-Physics, Inc., Mountain View, CA) was operated at 852 nm with Styryl 9 (Exciton Inc., Dayton, OH) as the laser dye. The dye laser output was frequency doubled to 426 nm in a 3 mm $LiIO_3$ crystal and the remaining 852 nm light blocked with a model 4-97 color filter (Corning Glass Works, Corning, NY). Data were collected at 5 ps/channel to a maximum of ~4,000 counts in the peak channel. The sample was flowed through the 0.3 mm pathlength fluorescence cuvette at 0.1 ml/min to avoid photodegradation. The response time of the instrument was 60–65 ps.

As will be shown below, the lifetime of the main component of the PYP fluorescence emission is quite short (≈12 ps; a total of three decay components was resolved). Although the data statistics were insufficient to accurately determine such a short lifetime in single decay measurements, a global analysis procedure was used (Causgrove et al., 1990) which greatly improves the accuracy of the measurement (Knutson et al., 1983). This was tested using simulated data with a main component having an 11 ps lifetime and a similar number of counts in the peak channel (4,000) as was obtained with an actual PYP sample. Whereas the 11 ps lifetime could not be resolved using a single decay, with global analysis of four decays, values of 11.4, 11.4, and 11.8 ps were obtained for the fast decay process in three separate trials on separate data sets. It should be noted, however, that this artificial data test assumes that the correct number of exponentials in the decay is known. It is always possible that there are more than the three that were resolved. If this is the case, then photon counting experiments will not be able to separate this situation from a single fast decay. Finally, it should be noted that we have previously determined a 16 ps lifetime with this instrument, which was supported by streak camera measurements, and that we have measured the decay of the dye pinacyanol, which consistently gives a lifetime of 10 ps. Although both of these were obtained using 10,000 counts in the peak channel, they show that a 12 ps decay is resolvable with the present apparatus.

RESULTS AND DISCUSSION

The fluorescence spectrum of the *E. halophila* PYP was determined using excitation at 445 nm and is shown in Fig. 1. The emission maximum was at 495 nm. The fluorescence quantum yield was found to be $1.4 \pm 0.1 \times 10^{-3}$. We had previously determined that the quantum yield for photochemical bleaching of the protein was 0.64 (Meyer et al., 1989). Therefore, it is not surprising that PYP is not highly fluorescent. Nevertheless, it is possible to measure the lifetime of the excited state by using fluorescence as a probe.

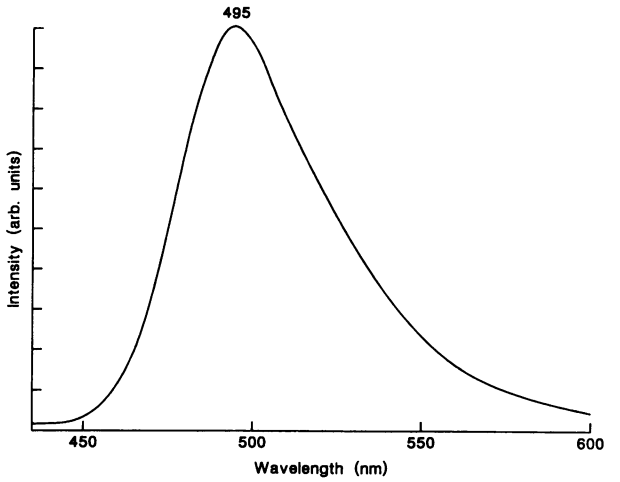

FIGURE 1 Fluorescence emission spectrum for *E. halophila* PYP. Excitation was at 445 nm.

Picosecond kinetics of fluorescence decay were measured with excitation at 426 nm. Emission was followed at 485, 500, and 510 nm, with nearly identical results. A typical decay curve is shown in Fig. 2. A global analysis

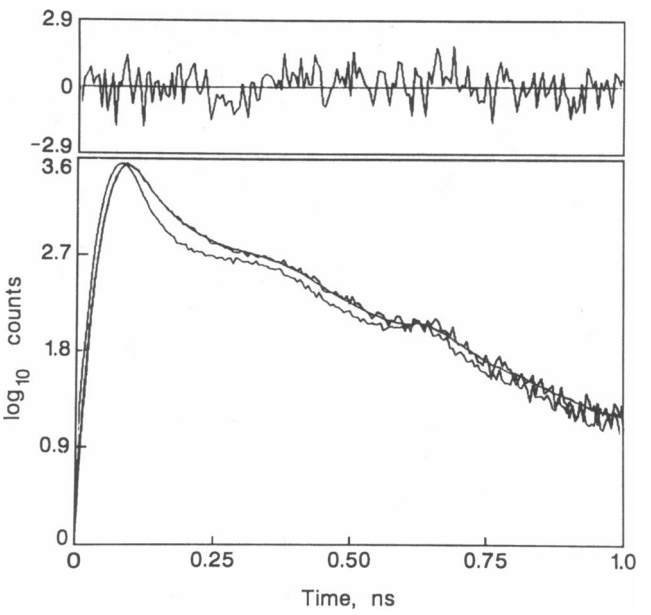

FIGURE 2 Picosecond fluorescence decay kinetics for *E. halophila* PYP. Excitation was at 426 nm and emission was monitored at 510 nm. The data were fit with a sum of three exponentials with lifetimes given in the text. The fluorescence decay and fitted curve are shown above the instrument response. At the top of the plot is the weighted residual between fitted and decay curves.

(Causgrove et al., 1990) of four decay curves indicates that 89.9% of the decay occurs with a lifetime of 11.7 ± 0.5 ps, 10.0% at 62.8 ± 2.3 ps, and a scant 0.2% at 339 ± 20 ps. The latter process is probably insignificant, considering the signal-to-noise levels in the latter part of the decay curve and the small amplitude. The intrinsic lifetime, calculated from the absorption spectrum using the following approximate formula:

$$1/\tau_0 = 3 \times 10^{-9}\, \nu_{max}^2\, \Delta\nu\, \epsilon_{max},$$

where $\Delta\nu$ is the half bandwidth, is 4.7 ns, and the actual lifetime calculated from the fluorescence quantum yield is 6.3 ps. It should be noted that other broadening mechanisms are ignored in this analysis, and thus the calculated value is a lower limit. The agreement between the measured and the estimated lifetimes for the excited state is satisfactory, considering the assumptions involved in the estimation and the difficulty in making measurements on such a fast time scale. This suggests that a single quenching process, which is presumably associated with the primary photochemical event, is primarily responsible for the decay of the first excited singlet state of PYP.

We had previously measured the kinetics for bleaching ($k = 7.5 \times 10^3\ s^{-1}$) and recovery ($k = 2.6\ s^{-1}$) and had determined that a partially bleached and red-shifted intermediate was formed in a time shorter than 10 ns (Meyer et al., 1989). There may be additional intermediates which occur between 12 ps and 10 ns, although we presently have no data bearing on this point. Fig. 3 summarizes our present knowledge of the PYP photocycle.

The lifetime of the excited state in bacteriorhodopsin is between 3 and 15 ps depending on conditions of measurement (Stoeckenius et al., 1979), and is thus remarkably similar to that of PYP in spite of the lack of similarity of the protein structures. Early intermediates in the bacteriorhodopsin photocycle are red shifted relative to the resting state, and the kinetics of bleaching and recovery are analogous to those of PYP. The sensory rhodopsins of *Halobacterium* are even more similar to PYP in native absorption spectrum and in kinetics of bleaching and recovery. Although the chromophore of PYP is still unknown, it is likely to be an aliphatic hydrocarbon with multiple conjugated double bonds such as in retinal, the chromophore in the bacterial rhodopsins. Our experiments suggest that the photochemical mechanisms of these presumably different chromophores are probably similar.

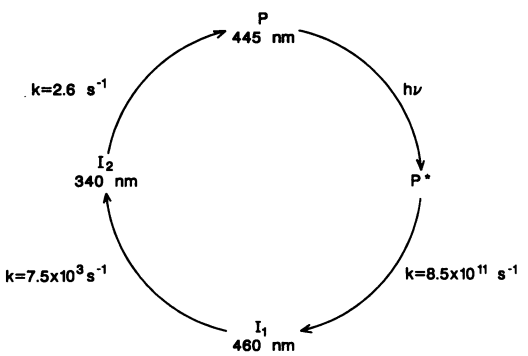

FIGURE 3 The *E. halophila* PYP photocycle as adapted from Meyer et al. (1989). There may be additional intermediates, which occur between 12 ps and 10 ns, but no measurements have yet been made within this time domain.

Measurements similar to those reported have recently been made with stentorin, the negative phototactic receptor in the ciliated protozoan *Stentor coeruleus* (Song et al., 1990). The chromophore of stentorin II is totally unrelated to that of PYP or bacteriorhodopsin, and no photocycle has yet been found for this pigment. The primary photoprocess in stentorin is thought to be proton dissociation, and thus the mechanism is probably unrelated to that in bacteriorhodopsin, which is known to undergo a primary *cis-trans* isomerization and is not involved in phototaxis. Stentorin II is membrane bound and weakly fluorescent, but exhibits a 10 ps fluorescence decay lifetime which is remarkably similar to that of PYP. As Song et al. (1990) point out, such a short-lived excited state is essential for efficient photobiological transduction.

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