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Two-photon-induced fluorescence from the phycoerythrin protein

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Two-photon-induced fluorescence is observed from the photodynamic phycobiliprotein phycoerythrin. Temporal, spectral, and intensity-dependent properties of the two-photon-induced fluorescence emission from phycoerythrin excited by a 1.06-μm laser beam are reported. The measured two-photon absorption cross section of phycoerythrin is an order of magnitude larger than that of Rhodamine 6G. The potential applications of phycobiliproteins for two-photon-induced fluorescence for microscopy of three-dimensional biological samples and three-dimensional optical memory are discussed.

Key words: phycobiliprotein, two-photon excitation, fluorescence, biosensors.

1. Introduction

Two-photon-induced fluorescence has been a useful spectroscopic tool to elucidate the molecular electronic structure of excited states. Recently it has received considerable attention because of the potential for using two-photon-induced fluorescence for biomolecular imaging with three-dimensional (3-D) resolution.[1,2] Two-photon-induced fluorescence is a nonlinear process in which the fluorescence emission depends quadratically on the excitation laser intensity. As a result the fluorescence signal from two-photon excitation is dominated by the most intense region of the excitation beam. This nonlinear process provides an optical sectioning effect that allows for fluorescence imaging with 3-D resolution without using the confocal configuration. Besides the inherent 3-D resolution obtained, two-photon fluorescence imaging is background free and the photobleaching of the fluorescent molecules is minimized. However, in order to use such a technique for imaging, it is necessary to find fluorescing tag molecules that possess high two-photon-induced fluorescence efficiency, are relatively stable, and exhibit minimum interference with the biological system to be imaged.

Phycobiliproteins are biomolecular assemblies located on the outer thylakoid membranes of marine algae. They are light-transducing proteins that harvest and funnel ambient light into the photoreactive center to drive photosynthesis.[3,4] The phycobiliproteins [phycoerythrin (PE), phycocyanin, and allophycocyanin] are organized as stacked discs in the phycobilisome, each with a distinct region of relatively narrow absorption in the visible spectrum. In this unique arrangement phycobiliproteins are able to efficiently absorb and transfer light energy, through a Forster mechanism, to chlorophyll for photosynthesis with greater than 90% efficiency.[3]

The individual phycobiliproteins possess interesting optical properties and are widely used today as fluorescent markers in biochemical and biomedical research.[5] For example, the outermost phycobiliprotein from the thylakoid membrane, PE, is a bulky, water soluble protein that displays very intense fluorescence (more than 20 times larger than fluorescein) with a high quantum yield and a large Stokes shift (2810 cm^{-1}, which is a factor of 2.7 larger than that of fluorescein).[5] Although single-photon excitation of PE is already widely used as a fluorescence tag for bimolecular research, to date no studies involving two-photon-induced fluorescence from phycobiliproteins have been reported. In this paper we report the first observation of two-photon-induced fluorescence from phycobiliproteins. Temporal, spectral,

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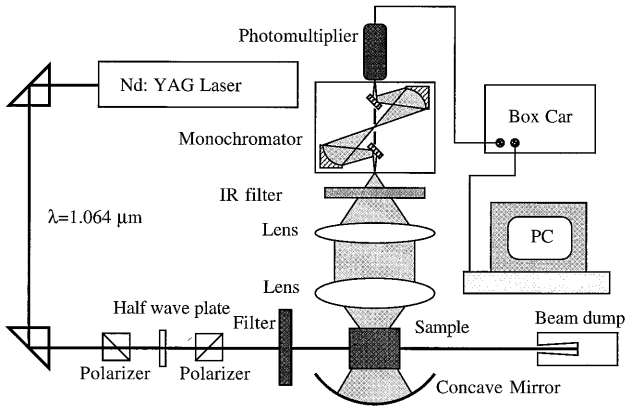

Fig. 1. Schematic diagram of the experimental setup for measuring the two-photon-induced fluorescence spectrum of phycoerythrin.

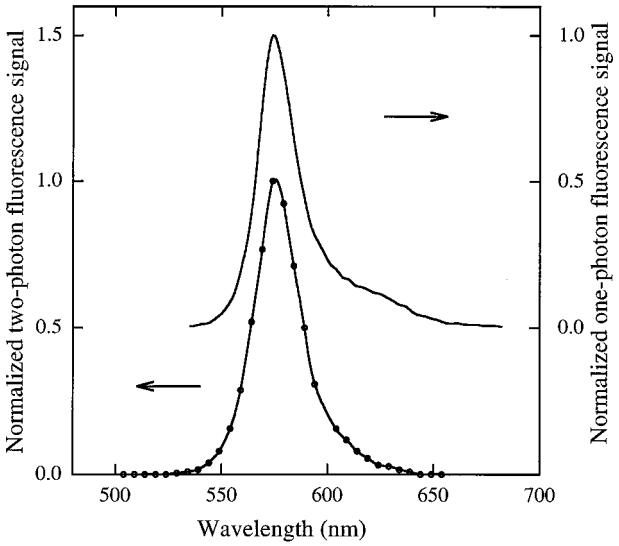

Fig. 2. Fluorescence spectra of PE in buffer solution. The lower curve is two-photon-induced fluorescence spectrum excited by a 1.06-μm laser beam. The upper curve is one-photon-induced fluorescence spectrum excited at 532 nm.

and intensity-dependent properties of the two-photon-induced fluorescence emission from phycoerythrin excited by a 1.06-μm laser beam are investigated. The two-photon cross section of PE at 1.06 μm is measured and the potential applications of phycobiliproteins for two-photon-based 3-D fluorescence scanning and imaging of biological samples and two-photon 3-D optical memory are discussed.

2. Materials and Methods

R-phycoerythrin was purchased from Molecular Probes (Eugene, Oregon) and used without further purification. The protocol to incorporate PE into solgel glass was described previously.[6] The experimental setup to measure two-photon-induced fluorescence is shown in Fig. 1. A *Q*-switched Nd:YAG laser with a 30-Hz repetition rate and 10-ns pulse width was used as the light source. The 1.06-μm IR beam from a Nd:YAG laser was passed through the sample cell, and the two-photon-induced fluorescence was collected with a focusing mirror and a condenser. The intensity of the IR beam was adjusted with a quarter-wave plate insert between two polarizers. The two-photon-induced fluorescence passed through a monochromator and was measured by a photomultiplier tube. The scattered IR laser beam was blocked by a KG 3 filter, and the signal was averaged with a boxcar integrator and collected with a personal computer.

3. Results and Discussion

PE has a characteristic absorption band at 550 nm for light harvesting and a characteristic fluorescence band around 575 nm for energy transfer.[4] We first measured the two-photon-induced fluorescence spectrum excited by a 1.06-μm laser beam (Fig. 2). For comparison we also show the one-photon-induced fluorescence spectrum excited by a 532-nm light beam. The relative fluorescence spectral distributions from one- and two-photon excitation are very similar. In both cases, the fluorescence peak is located around 575 nm and the fluorescence bandwidth is approximately 25 nm. The principal difference in the two spectra is that the small shoulder around 620 nm in the one-photon-excited fluorescence spectrum is absent from that of the two-photon-excited fluorescence spectrum.

The observed two-photon-induced fluorescence from PE is very strong. The intensity dependence of the two-photon-induced fluorescence is shown in Fig. 3 where the relative fluorescence intensity is plotted as a function of the square of the 1.06-μm laser intensity. The open circles are the measured fluorescence data, and the solid line is a linear fit. The linear fit of the data in Fig. 3 shows that the fluorescence signal is proportional to the square of the laser intensity, which indicates a two-photon-excitation

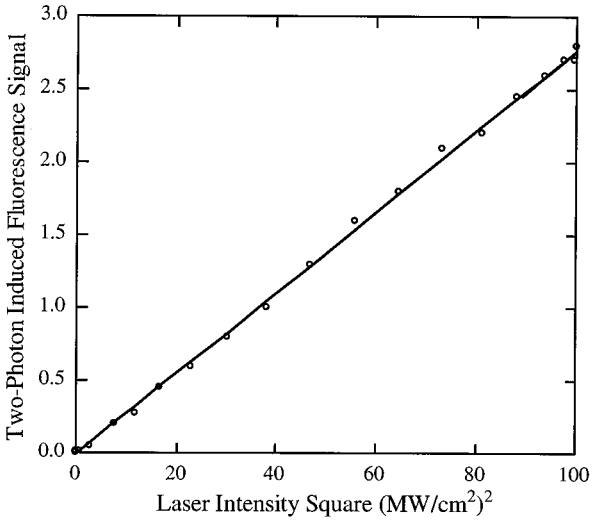

Fig. 3. Fluorescence signal of PE as a function of the square of the 1.06 μm-laser intensity.

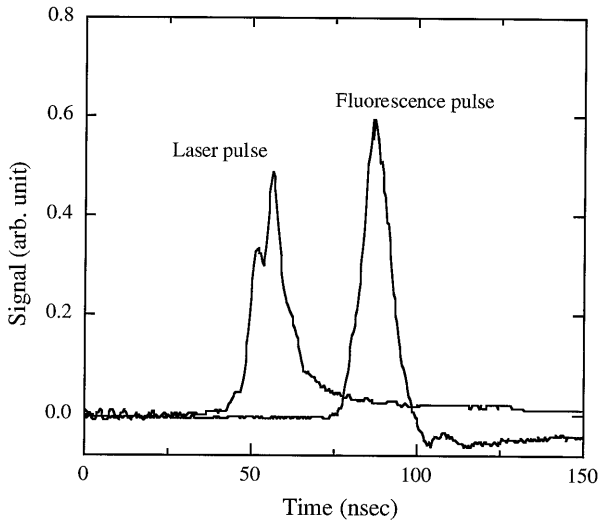

Fig. 4. Temporal profile of the two-photon-induced fluorescence. The curve on the left is the waveform of the laser beam, and the curve on the right is the waveform of the two-photon-induced fluorescence.

mechanism. Although deviation of the square law has been observed for a number of dyes in two-photon-induced fluorescence,[7] no deviation of the square law is observed for PE in our experiment.

To investigate the temporal behavior of the two-photon-induced fluorescence, a fast silicon diode (1-ns rise time) was used to detect the wave form of the 1.06-μm IR laser beam and a photomultiplier (2-ns rise time) was used to detect the two-photon-induced fluorescence. The wave form of the laser beam and the fluorescence signal is then recorded with a 300-MHz digital oscilloscope (HP 54510B) as shown in Fig. 4. The curve on the left in Fig. 4 is the waveform of the 1.06-μm laser pulse, and the one on the right is the waveform of the fluorescence signal from PE. The full width at half-maximum (FWHM) of the laser pulse is approximately 10 ns, and the FWHM for the two-photon-induced fluorescence is approximately 9 ns. This result indicates that two-photon-induced fluorescence from PE is a fast process and that both the rise and the decay times are less than the time resolution of our measuring system, which is of the order of a few ns.

The two-photon cross section was measured with Rhodamine 6G (R6G) used as a standard because the two-photon-absorption cross sections of R6G, which are wavelength dependent, have been characterized.[7–9] An expression for the two-photon-induced fluorescence signal F_i is given by[10]

$$F_i = K(\Phi_i/2)n_i\delta_i l I^2,$$

where Φ_i is the quantum yield, which has to be divided by a factor of two because two photons have to be absorbed for each photon emitted; n_i is the fluorophore number density; δ_i is the two-photon-absorption cross section; l is the path length; I is the flux of incident photons; and K is a dimensionless constant that takes into account the geometry of the optical setup, the efficiency of the photon collection system, and the correction for self-absorption. By measurement and comparison of the relative signal of the two-photon fluorescence emission from PE and R6G under identical experimental conditions, the two-photon cross section of PE can be determined.

In our experiment to determine the two-photon cross section, we diluted solutions of PE and R6G to the same optical density (0.25 in 10-mm path) in two identical semimicrocells. The low concentration and identical optical density minimize the difference in self-absorption corrections. The identical optical setup ensures that path length l and photon flux I are identical. The solid angle of collection, which affects the constant K, is different because of solvents with different refractive indices were used for R6G and PE.[11] However, because the difference in refractive index is small between water and ethanol (1.333 and 1.359, respectively), this difference is negligible. Although fluorescence emission spectra of PE and R6G peak at 575 and 550 nm, respectively, the spectral response of the light collection system near these two peaks is relatively flat. The relative value of constant K for PE and R6G can be calculated from the spectral response of the photomultiplier. The ratio of the two-photon cross section of PE and R6G is therefore given by

$$\delta_{PE}/\delta_{R6G} = (K_{R6G}/K_{PE})(\Phi_{R6G}/\Phi_{PE})(n_{R6G}/n_{PE})(F_{PE}/F_{R6G}).$$

Because the second harmonic of the 1.06-μm laser is within the one-photon absorption band of PE and R6G, we assume that the two-photon-induced fluorescence quantum yield is similar to that of one-photon-induced fluorescence. Using the published values[5] of quantum yields of PE and R6G ($\Phi_{R6G} = 0.82$, $\Phi_{PE} = 0.90$) and measured fluorescence signals under identical experimental conditions, we determined the ratio of two-photon cross sections for PE and R6G at 1.06 μm to be

$$\delta_{PE}/\delta_{R6G} = 25 \pm 2.$$

It should be pointed out that in the above calculation we have not taken into account the local field correction.[12] However, because the refractive indices of water and ethanol are similar, the effect of local field correction is expected to be small. Using the value of the two-photon cross section of R6G determined by Hermann and Ducuing[7] ($\delta_{R6G} = 12.9 \pm 6 \times 10^{-50}$ cm^4 s), we calculated the two-photon cross section of PE at 1.06 μm to be

$$\delta_{PE} = 322 \pm 110 \times 10^{-50}\ (\text{cm}^4\ \text{s}).$$

The large two-photon cross section of PE measured at 1.06 μm clearly suggests that PE is a very efficient molecular tag for two-photon fluorescent microscopy.

Three-dimensional imaging of biological samples plays a crucial rule in biomedical and biochemical research. Currently, confocal microscopy is being widely used for fluorescence imaging of biological samples with 3-D resolution. However, one problem

encountered with the fluorescence image using the confocal technique is the photobleaching of the fluorescent probes. Although phycoerythrin is known as a light harvesting protein and is stable under ambient light conditions, it has been found to photobleach under intense visible light illumination. The stability of PE toward intense two-photon excitation is important for the application of PE for two-photon fluorescence microscopy. In addition, PE may function well as a two-photon 3-D optical memory material. 3-D optical memories that employ two-photon processes with two intersecting beams for writing and reading have already been proposed.[13–16] Two-photon fluorescence is one of the methods used to access data in a 3-D optical memory.

Although a number of organic molecules have been proposed for such an application,[13] materials based on biological molecules offer the advantage of tailorability and optimization of properties through genetic engineering. This has been illustrated by Hampp *et al.*[17] with bacteriorhodopsin-based biomaterials, where specifically designed mutants were found to improve device performance. The potential application of PE for 3-D optical memories has been demonstrated by the successful encapsulation of PE in a transparent solgel glass and the observation of an intense two-photon-induced fluorescence from the PE-doped solgel glass.[6] However, when PE is used for these applications, its stability towards photodegradation becomes a critical issue. Although PE is known as a light-harvesting protein and is stable under ambient light conditions, it has been found to denature under intense visible light illumination.[6] However, to our knowledge no studies on the photostability of PE under IR illumination have been reported. We tested the photostability of PE under illumination with an intense 1.064-μm laser pulse. This was accomplished by monitoring the two-photon-induced fluorescence when the sample was illuminated with a 1.06-μm laser pulse. To avoid the diffusion of the sample and ensure that the pulsed laser illuminated the same PE sample, the protein was entrapped in a wet silica gel by a solgel technique.[6] Figure 5 shows the two-photon-induced fluorescence signal as a function of the number of times that the sample was illuminated with the pulsed laser beam. The peak intensity of the illuminated laser beam is approximately 35 MW/cm^2. No decrease in fluorescence signal was observed after the sample was illuminated more than 5×10^5 times with an intense pulsed IR laser. Although a much higher laser intensity is necessary to measure the photobleaching rate, our result indicates that PE is stable under intense IR illumination for the peak light intensity of at least 35 MW/cm^2. The large observed two-photon-induced fluorescence, enhanced stability, and the ability to specifically tailor its properties make PE a promising material for 3-D two-photon microscopy and optical memories.

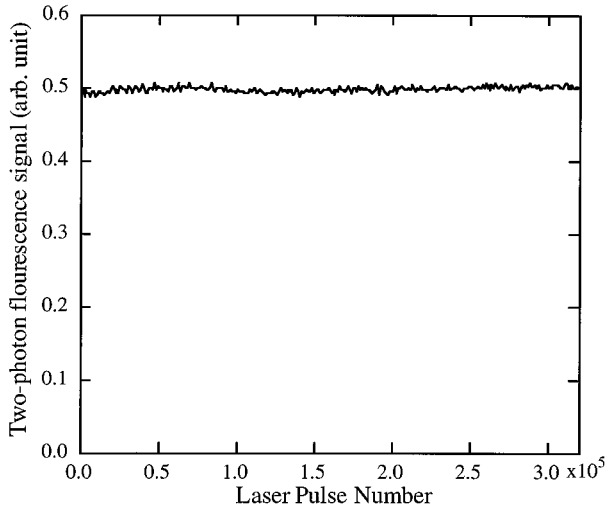

Fig. 5. Stability of PE toward photodegradation of 1.06-μm laser excitation.

4. Conclusion

We have observed two-photon-induced fluorescence from a phycobiliprotein. PE exhibits very strong two-photon-induced fluorescence. This observed two-photon-induced fluorescence, combined with its inherent photochemical properties, high stability, and the ability to tailor this system through genetic engineering, make this biomolecule promising for potential applications in biomolecular sensing, imaging and information processing and storage.

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