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Two-photon excitation of a phytofluor protein

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Abstract

Phytofluors are highly fluorescent proteins in which the chromophore in a phytochrome is replaced with phycoerythrobilin (PEB), the pigment precursor of the cyanobacterial light harvesting protein phycoerythrin. We examined the fluorescence spectra of the N-terminal region of the cyanobacterial phytochrome 1 from cyanobacterium *Synechocystis* sp. Pcc 6803 bound to PEB. This protein, Cph1(N514)-PEB, displayed a good two-photon cross-section of 20–30 GM for excitation at 792 nm. This phytofluor also exhibits a high fundamental anisotropy at most practical two-photon excitation (2PE) wavelengths from 700 to 900 nm. Identical lifetimes and correlation times with one and 2PE indicates that the phytofluor is not adversely affected by the intensities needed for 2PE. The one-photon absorption extends well beyond the absorption spectrum and even beyond the emission spectrum to 700 nm. The phytofluor thus appears to be a suitable probe for 2PE and/or cellular imaging. © 2002 Published by Elsevier Science B.V.

Keywords: Green fluorescent protein; One-photon excitation; Two-photon excitation

1. Introduction

Fluorescence detection is often performed using the extrinsic fluorophores fluorescein, rhodamine, and similar species. Because these fluorophores have to be covalently attached to their targets, which is difficult to control, there has been an increasing interest in the use of naturally fluorescent proteins as intracellular probes. This interest intensified following the introduction of the highly fluorescent phycobiliproteins as probes for high sensitivity detection, single particle detection and medical assays [1–5]. More recently, it was found that green fluorescent protein (GFP) oxidizes spontaneously to form a highly fluorescent product [6], resulting in the widespread use of GFP as a tag for gene expression, cellular imaging and ion sensing within living cells [7–10]. The discovery of red and yellow fluorescent proteins from coral [11–13], suggests that additional intrinsically fluorescent, GFP-related proteins will soon be identified.

A new class of plant-derived fluorescent bili-protein was recently reported, the termed phytofluors [14]. Phytofluors are derived from the phytochromes, which are photoreceptor proteins in plants that regulate such light modulated processes as seed germination, flowering, shade avoidance and intracellular organelle movement [15,16]. Plant phytochromes are soluble homo-dimers which consist of two domains a globular N-terminal light-sensing domain and a C-terminal signaling domain that is volutionarily related to two component histidine kinases of prokaryotes [16]. The N-terminal domain contains a covalently bound linear tetrapyrole (bilin) pigment, phytochromobilin (PσB) or phycocyanobilin (PCB) [17,18]. Absorption of red or far-red light results in photointerconversion of the bilin pigment that are accompanied by conformational changes in the protein [19,20]. Because of the rapid isomerization of the bilin pigment, phytochromes are weakly fluorescent, with quantum yield less than 10^{-3} at room temperature [21]. The discovery that recombinant apo-phytochromes spontaneously forms complexes with ethylidene-containing linear tetrapyrroles [22–24] led to the discovery of highly fluorescent phycoerythrobilin (PEB) adducts of apophytochromes called phytofluors [14]. Phytofluors are brightly fluorescent, possess large molar absorption coefficients up to 165,000 M^{-1} cm^{-1}, display quantum yields in excess of 0.7, and exhibit high photostabilities. Apophytochromes can be expressed in cells which then become fluorescent upon incubation with exogenous bilin pigments [14].

Abbreviations: GFP, green fluorescent protein; 1PE, one-photon excitation; 2PE, two-photon excitation; FD, frequency-domain; PEB, phycoerythrobilin

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Multi-photon excitation has become a method of choice for cellular imaging because of localized excitation, pseudo-confocal imaging and minimal photodamage [25–27]. Two-photon excitation (2PE) has been used to image GFP [28,29] and an initial report has appeared on 2PE imaging of *E. coli* cells expressing a red fluorescent protein [30]. It is therefore likely that the phytofluors will also be useful for multi-photon microscopy. Here we report the fluorescence characteristics of a phytofluor derived from the bilin binding domain of cyano bacterial phytochrome 1, i.e. Cph1(N514)-PEB, using both 1PE and 2PE of methodologies. Phytofluor Cph1(N514)-PEB represents the 514 amino terminus of Cph1 sp.Pcc 6803 complexed with PEB. Phytofluor Cph1(N514)-PEB is monomeric in solution and spectrally indistinguishable from the phytofluor derived from the full length Cph1 apoprotein [14,31].

2. Materials and methods

Phytofluor Cph1(N514)-PEB was prepared as described previously [14]. Solutions were in 50 mM Tris buffer, pH 8, 1 mM EDTA and 25% ethylene glycol, with a concentration of 8.2 μM based on a molar extinction coefficient of 85,400 M^{-1} cm^{-1} at 579 nm.

Multi-photon excitation was performed using the fundamental output of a femtosecond Ti:Sapphire laser (Spectra Physics, Tsunami), at 792 nm. The laser system was equipped with pulse selector providing repetition rate 4 MHz, pulse width of 90 fs and average power of about 20 mW. The excitation beam was focused on the sample with 2 cm best form lens. The 1PE was performed using the frequency-doubled output of a picosecond Py2 dye laser (345–385 nm), the frequency-doubled Ti:Sapphire laser, or the fundamental output of a rhodamine 6G dye laser (570 nm). Cavity dumped dye lasers provided 7 ps pulses with repetition rate of 3.86 MHz and average power of about 50 mW. Frequency-domain (FD) intensity decay were measured using magic-angle polarization and instrumentation described previously [32,33]. Intensity decays were analyzed by non-linear least squares [34] in terms of the multi-exponential model

$$I(t) = \sum_i \alpha_i \exp\left(\frac{-t}{\tau_i}\right) \quad (1)$$

where α_i are the pre-exponential factor, $\sum \alpha_i = 1.0$, and τ_i are the decay times. The fractional intensity associated with each decay time is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (2)$$

FD anisotropy decays were analyzed in terms of the multi-correlation time (θ_i) model [32]

$$r(t) = \sum r_{0k} \exp\left(\frac{-t}{\theta_k}\right) \quad (3)$$

where r_{0k} is the amplitude of each correlation time decay and $\sum r_{0k} = r_0$ is the time-zero anisotropy. This value is expected to be equivalent to the value observed in frozen solution where the chromophore does not rotate during the excited state lifetime. For intensity and anisotropy decay measurements the emission was observed through a 600 nm interference filter with a 20 nm bandpass.

3. Results and discussion

Fluorescence emission spectra of phytofluor Cph1(N514)-PEB are shown in Fig. 1 for excitation at the 580 nm absorption maximum and for excitation at 792 nm. An identical emission spectrum was observed for excitation at 396 nm, half of the long excitation wavelength (not shown). At 792 nm excitation the emission intensity depends quadratically on the incident power (insert), demonstrating two-photon absorption. These identical emission spectra demonstrate that emission occurs from the same excited state, independent of the excitation wavelength or the mode of excitation. In order to test the photostability of the sample upon 792 nm excitation, we illuminated the

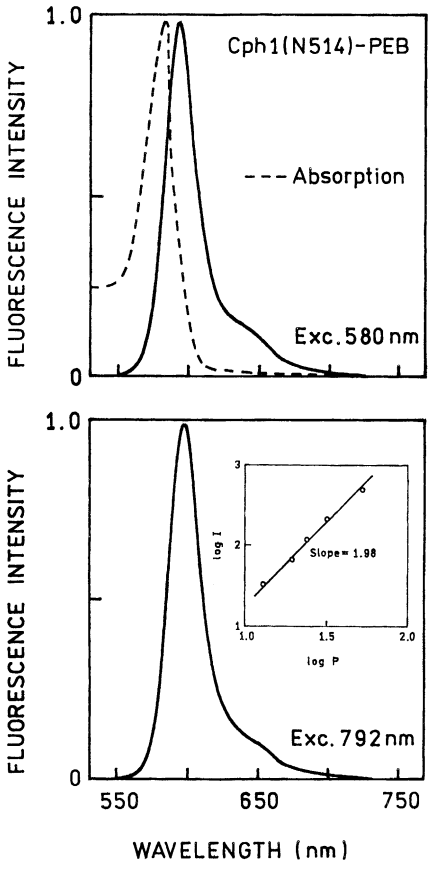

Fig. 1. Fluorescence emission spectra of phytofluor Cph1(N514)-PEB in 50 mM Tris–HCl pH 8 buffer, 1 mM EDTA and 25% ethylene glycol. Top: 1PE, bottom: 2PE at 792 nm using a Ti:Sapphire laser. Insert shows the power dependence of the emission intensity.

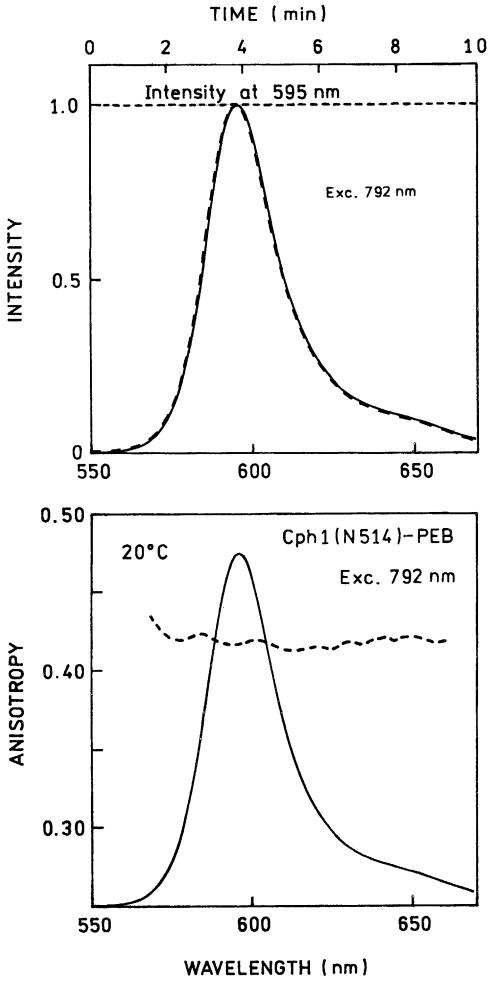

Fig. 2. Photostability of phytofluor Cph1(N514)-PEB upon 792 nm illumination (top). The spectra were measured before (—) and after 10 min exposure (– – –). The steady-state emission anisotropy of phytofluor Cph1(N514)-PEB with 2PE (– – –) (bottom).

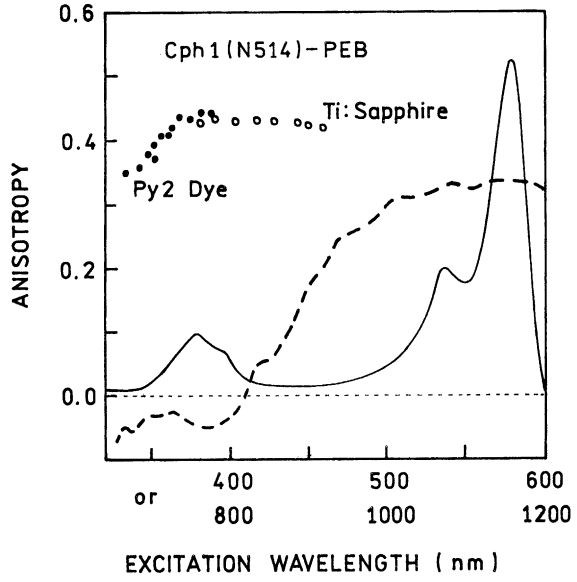

Fig. 3. The 1PE and 2PE anisotropy spectra of phytofluor Cph1(N514)-PEB at 20 °C (symbols: ●, ○). 1PE anisotropy (dashed line) and fluorescence excitation spectra are shown (solid line).

sample for over 10 min, monitored the fluorescence intensity at 595 nm and remeasured the fluorescence spectrum (Fig. 2, top). There was no evidence of any change in intensity or spectral distribution. The time needed for spectral measurement was about 1 min. Also, the absorption spectrum of the sample, before and after 10 min illumination at 792 nm remained unchanged. We examined the emission anisotropy for excitation at 792 nm (Fig. 2, bottom). The steady-state anisotropy values (r) are constant across the emission spectrum, demonstrating emission from a single excited state. Moreover, the anisotropy exceeds 0.4, the upper limit for one-photon excitation (1PE) of an isotropic sample [35] demonstrating that the emission induced by 792 nm excitation is due to multi-photon absorption [36].

We examined the excitation anisotropy spectra for phytofluor Cph1(N514)-PEB (Fig. 3). The anisotropy with 1PE remained high from 450 to 600 nm. It is interesting to notice that the anisotropy remained high for wavelengths below the 580 nm absorption maximum. This suggests the excitation even in the weakly absorbing region from 450 to 500 nm is due to a transition to the lowest singlet state (S_1). For shorter wavelength excitation the anisotropy becomes negative, which indicates the transition moment for the $S_0 \rightarrow S_2$ transition is nearly perpendicular to the $S_0 \rightarrow S_1$ transition moment. Surprising by different results were obtained for the excitation anisotropy spectra with 2PE. For wavelengths regions where the anisotropy is low or negative for 1PE, the anisotropy is both high and positive for 2PE (Fig. 3). This result indicates that two-photon absorption around 800 nm results in a $S_0 \rightarrow S_1$ transition. Thus the two-photon absorption spectrum of Cph1(N514)-PEB differs from the one-photon absorption spectrum. That is, the two-photon absorption spectrum is not the same as the one-photon spectrum translated and expanded to the longer wavelength region.

We next measured the FD intensity decay with 1PE and 2PE protocols (Fig. 4). As shown in Table 1, the intensity decays were mostly a single exponential irrespective of the

Table 1
Intensity decays analyses of phytofluor Cph1(N514)-PEB measured at different excitation wavelengths

Excitation (nm)	$\bar{\tau}$ (ns)[a]	α_1	α_2	τ_1 (ns)	τ_2 (ns)	χ_R^{2b}
570	2.91	0.251	0.749	1.03	3.12	1.9 (48.1)
792	3.03	0.192	0.808	1.50	3.21	2.4 (10.9)
396	2.95	0.372	0.628	1.36	3.34	0.9 (44.4)
685	2.86	0.523	0.477	1.80	3.45	1.3 (24.6)

[a] $\bar{\tau} = \sum_i f_i \tau_i$, $f_i = \alpha_i \tau_i / \sum \alpha_i \tau_i$.

[b] χ_R^2 is the goodness-of-fit parameter calculated with assumed uncertainties in the phase angle of 0.3° and in the modulation of 0.004 [32]. The values in parentheses are the χ_R^2 values for the single decay time fits.

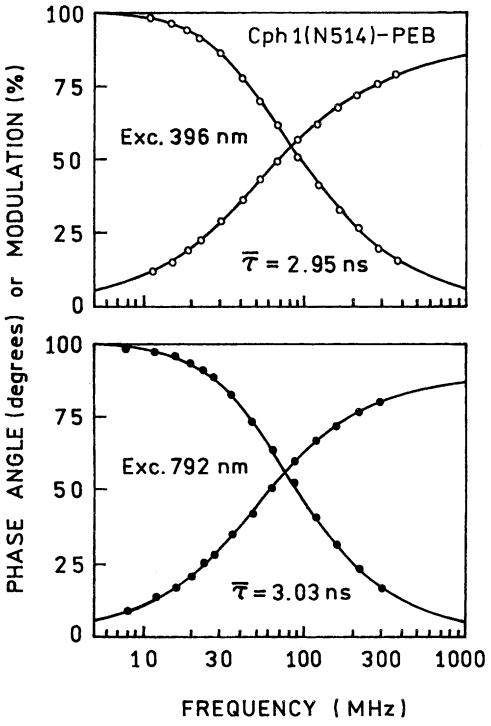

Fig. 4. FD intensity decays of phytofluor Cph1(N514)-PEB with IPE (top) and 2PE (bottom) excitation, protocols.

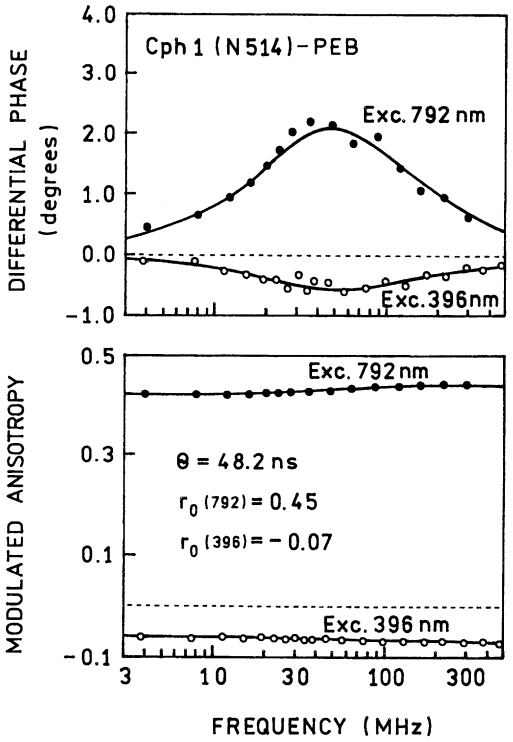

Fig. 5. FD anisotropy decays of phytofluor Cph1(N514)-PEB with 1PE (○) and 2PE (●) protocols analyzed simultaneously.

excitation wavelength from 396 to 792 nm and lifetimes were in good agreement with those reported previously [14]. Thus, phytofluor Cph1(N514)-PEB obeys Kasha's rule by emitting only from the lowest single state, indicating rapid relaxation from higher electronic/vibrational levels to the ground state [37].

In contrast to intensity decays, FD anisotropy decays were strongly dependent on the excitation wavelength (Fig. 5). While a single rotational correlation time was observed at all excitation wavelengths (Table 2), the different shape and magnitude of the different phase and modulated amplitude data reflect different time-zero anisotropies in solution. The FD data obtained for four excitation wavelengths were analyzed globally for a single correlation time, but different time-zero anisotropies (Table 2). The goodness-of-fit for the global analysis indicates that a single correlation time is adequate to describe the FD data irrespective of the mode of excitation. This result demonstrates the protein structure is not perturbed by the intense 792 nm illumination used for 2PE. The recovered correlation time of 48.2 ns is longer than that expected for a hydrated sphere (0.2 g H_2O per g protein) with a molecular weight near 64,200, $\theta = 25$ ns. The longer correlation time for phytofluor Cph1(N514)-PEB suggests that the protein is elongated with an axial ratio near 2.5. We also estimated the two-photon cross-section for Cph1(N514)-PEB by comparison with RhB (Fig. 6). Assuming RhB has a cross-section of 150 GM at 792 nm [38,39], Cph1(N514)-PEB displays a 6-fold smaller cross-section, about 20–30 GM. One GM is equal to a two-photon cross-section of 10^{-50} cm^4 s per photon.

For the use of phytofluors with multi-photon excitation, it is important to know the wavelength range where the excitation shifts from one- to two-photon absorption. We evaluated this transition by examining the decrease in emission intensity for a 2.19-fold decrease in excitation intensity (Fig. 7). Excitation below 700 nm was shown to be a one-photon process, while excitation above 720 nm was a two-photon process. A similar transition was observed using the steady-state anisotropy (Fig. 8). The one-photon to two-photon transition

Table 2
Anisotropy decay analysis of phytofluor Cph1(N514)-PEB

Excitation (nm)	θ (ns)	r_0	χ^2_R [a]
570	46.0	0.355	0.9
792	48.8	0.447	1.3
396	28.0 [b]	−0.067	0.8
685	50.6	0.366	1.0
Global			
570		0.355	
792		0.447	
396		−0.065	
685	48.2	0.367	1.0

[a] χ^2_R is the goodness-of-fit parameter calculated with an assumed uncertainity of 0.3° in the differential polarized phase angle and of 0.007 in the ratio of polarized modulation amplitudes [32].

[b] This low value is due to reduced resolution with the low value of r_0, and is not thought to be a true value.

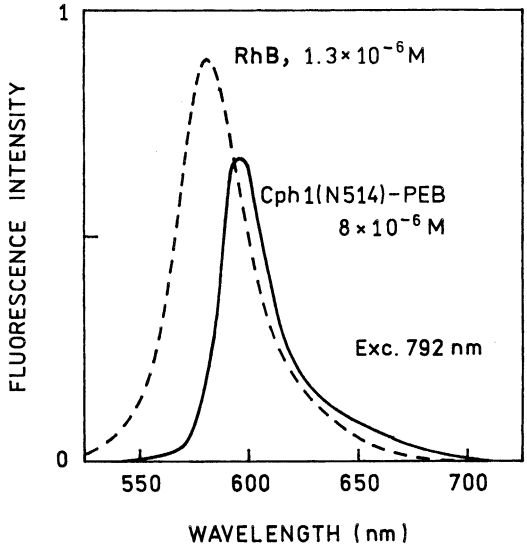

Fig. 6. Comparison of the fluorescence emission of phytofluor Cph1(N514)-PEB solution (optical density 0.7) with 1.3×10^{-6} M rhodamine B in ethanol (– – –). Assuming a molar absorption coefficient at 529 nm 85,400 M^{-1} cm^{-1} ($C = 8 \times 10^{-6}$ M), the two-photon cross-section is approximately 6-fold smaller than that of RhB; $\sigma_{792} = 20$–30 GM.

is centered at a surprisingly by long wavelength of 720 nm, which is well above the last absorption maximum near 580 nm, and even beyond the emission spectrum which is maximal at 590 nm with little emission above 650 nm (Fig. 2). Remarkably, phytofluor Cph1(N514)-PEB displays one-photon absorption at wavelengths much longer than its emission spectrum. This result prompted us to examine the excitation spectrum of Cph1(N514)-PEB (Fig. 9). This phytofluor displays a weak one-photon absorption centered

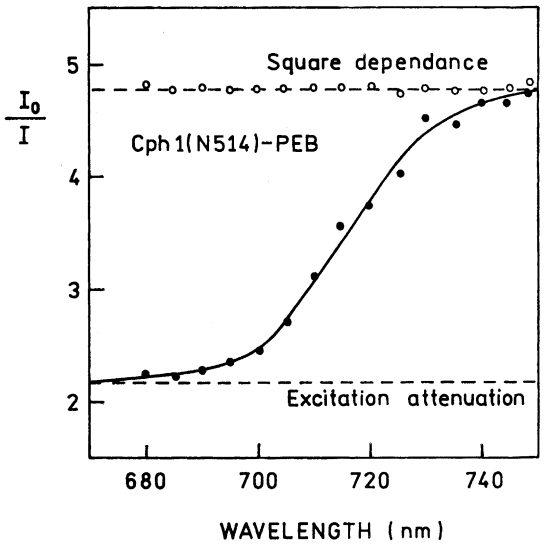

Fig. 7. Wavelength-dependent attenuation of emission intensity of phytofluor Cph1(N514)-PEB upon excitation attenuation of 2.19 (0.4 neutral density filter). I_0 and I are the emission before and after attenuation of the excitation, respectively.

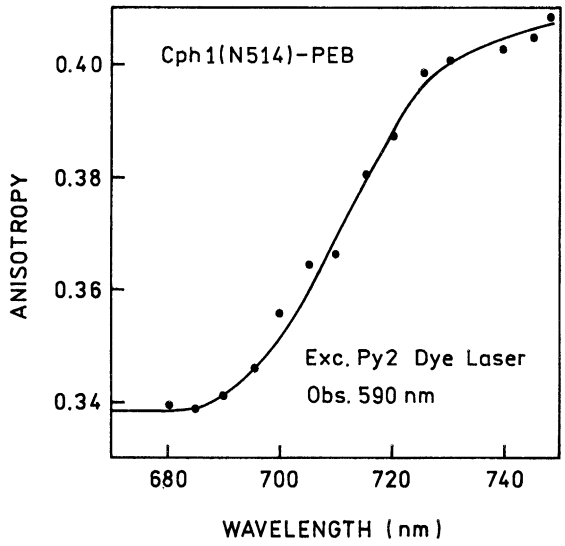

Fig. 8. Wavelength-dependent steady-state anisotropy of phytofluor Cph1(N514)-PEB.

near 640 nm, extending to 700 nm or longer. Excitation at 685 nm was clearly a one-photon process (Fig. 10), and shifted to a two-photon process at 750 nm. We also examined the temperature dependence of the excitation spectra for 560 and 645 nm excitation (Fig. 11). For 560 nm excitation the fluorescence emission intensity of N514-PEB decreased from 0–40 °C (top). While 685 nm excitation, the intensity of Cph1(N514)-PEB increases 0 to 40 °C (bottom). These effects are reversible upon reducing the temperature. These results suggest that the species absorbing at 640 nm is thermally populated. Additional studies are needed to clarify the nature of this long wavelength absorbing state.

Suppose one is required to excite the phytofluor in the one-photon to two-photon transition near 715 nm. The

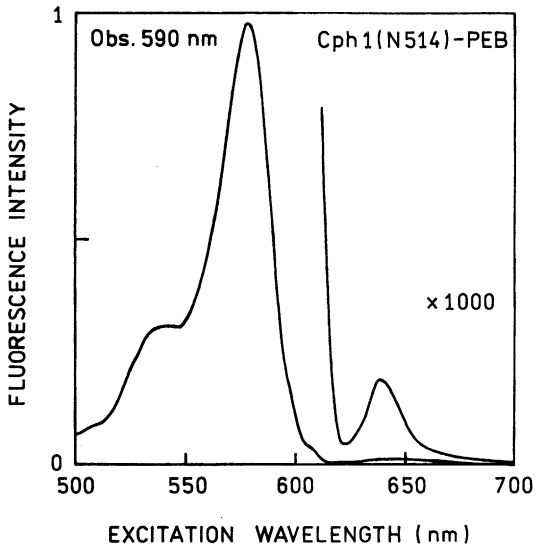

Fig. 9. The fluorescence excitation spectrum of phytofluor Cph1(N514)-PEB. The 1PE extends up to 700 nm.

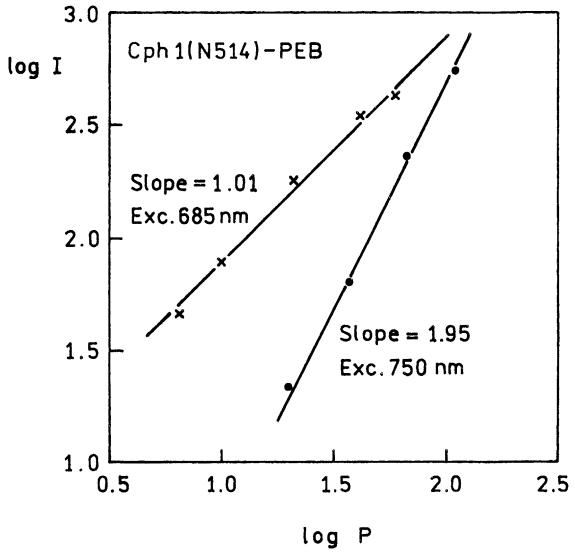

Fig. 10. The dependence of phytofluor Cph1(N514)-PEB emission intensity on excitation power. At 685 nm excitation, there is still one-photon absorption, while at 750 nm the excitation is due simultaneous two-photon absorption.

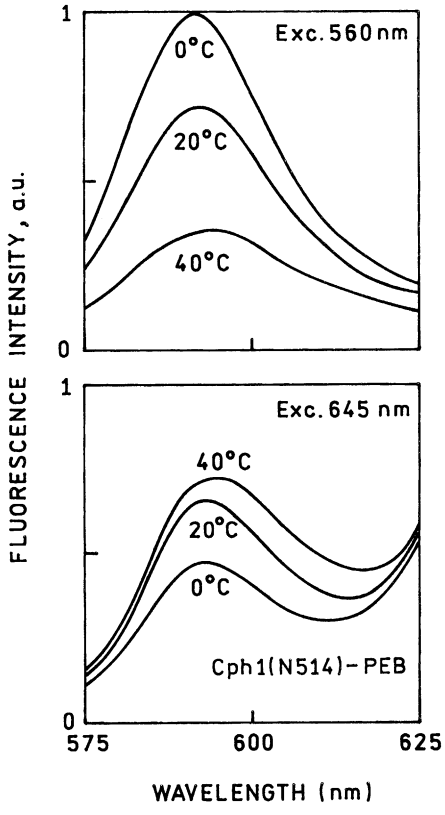

Fig. 11. Temperature dependence emission spectra of phytofluor Cph1(N514)-PEB fluorescence at 560 nm (top) and 645 nm (bottom) excitation. The rising intensity at long wavelengths in the lower panel is apparently due to scattered excitation light.

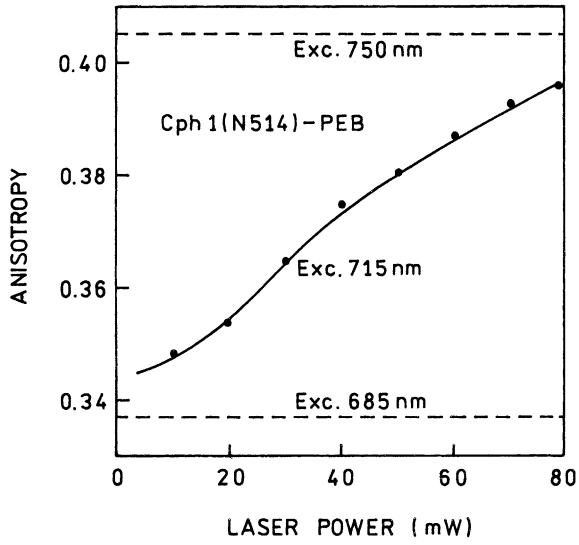

Fig. 12. Dependence of steady-state anisotropy of phytofluor Cph1(N514)-PEB Cph1-N514 adduct on laser power. At 715 nm excitation, the anisotropy (mode of excitation) depends on laser power.

intermediate anisotropy (Fig. 8) indicates that both one and two-photon absorption are occurring. At these wavelengths, the mode of excitation depends on the incident power. This can be seen from the dependence of the anisotropy on incident power (Fig. 12). This dependence suggests that the mode of excitation can be determined from the anisotropy, or conversely, that a constant anisotropy with varying incident power indicates pure 1PE or 2PE.

4. Conclusions

Phytofluor Cph1(N514)-PEB displays a good cross-section for 2PE about 6-fold less than that found for RhB. Except for the anisotropy, this phytofluor displays the same emission spectral properties independent of the mode of excitation. The transition between the modes of IPE–2PE occurred near 720 nm (Figs. 7 and 8) which is more than 100 nm red shifted from the emission maximum. Pure 2PE can be observed only for excitation wavelengths longer than 750 nm. The anti-Stokes 1PE responds differently to the temperature than short-wavelength excitation (Fig. 11), at higher temperatures the excitation is more effective. This is consistent with an assumption that anti-Stokes 1PE occurs from a thermally populated vibronic state.

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