Fluorescence Spectral Properties of Troponin C Mutant F22W with One-, Two-, and Three-Photon Excitation

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ABSTRACT We report the first measurements of protein fluorescence with three-photon excitation, using a mutant of troponin C (TnC) that contains a single tryptophan residue F22W. From the emission intensity dependence on laser power we determine that TnC F22W displays one-, two-, and three-photon excitation at 285, 570, and 855 nm, respectively. The emission spectra and intensity decays are identical for one-, two-, or three-photon excitation. The steady-state and time 0 anisotropies are distinct for each mode of excitation, but the correlation times were the same, suggesting that three-photon excitation of proteins can be accomplished without significant effects of the locally intense illumination. The excitation anisotropy spectrum from 830 to 900 nm displays only negative values, suggesting dominant excitation via the ^1L_b state of tryptophan from 830 to 900 nm.

INTRODUCTION

Time-resolved fluorescence is often used to study the structure and dynamics of proteins (Beechem and Brand, 1985; Demchenko, 1992; Jameson and Reinhart, 1989). For most proteins the emission is due mostly to the tryptophan residues, which are known to be highly sensitive to the local environment and the presence of nearby quenchers. With few exceptions (Lakowicz and Gryczynski, 1993; Lakowicz et al., 1992a, and references therein) most studies of the intrinsic fluorescence of proteins have been accomplished by using one-photon excitation at ultraviolet wavelengths.

In recent years the increasing availability of picosecond and femtosecond laser sources has allowed two-photon excitation of proteins (Lakowicz and Gryczynski, 1993; Lakowicz et al., 1992a) and other biopolymers (Lakowicz et al., 1992b; Lakowicz and Gryczynski, 1992). By two-photon or three-photon excitation we are referring to simultaneous absorption of two or three long-wavelength photons from the same laser beam. Hence the absorbed photons have the same wavelengths and polarization. With present technology two-photon excitation is best accomplished using the femtosecond pulses from mode-locked Ti:sapphire lasers. However, these lasers provide wavelengths from 700 to 1000 nm, with maximum output from 800 to 900 nm. Because the long-wavelength absorption of proteins ends at 300 nm, two-photon excitation of proteins is not possible with a Ti:sapphire laser, with its present lower wavelength limit near 700 nm.

In the present report we show that protein fluorescence can be excited by a three-photon process with the 855-nm output of a Ti:sapphire laser. For these studies we selected a mutant of troponin C that contains a single tryptophan residue F22W (She et al., 1996a). In this mutant the trp residue is located on helix A and is partially buried in the absence of bound Ca^{2+} at the two regulatory sites in the N-terminal domain. We compared the spectral properties of troponin C (TnC) F22W for one-, two-, and three-photon excitation. The similarity of the intensity decay times and anisotropy correlation times for each mode of excitation suggests that three-photon excitation can be accomplished without significant photochemical or thermal effects on the proteins.

MATERIALS AND METHODS

Preparation of troponin C mutant F22W

The construction of the expression vector and the production of TnC mutant F22W by site-directed mutagenesis will be described elsewhere (She et al., 1996b). Briefly, a cDNA encoding full-length TnC of chicken fast skeletal muscle was isolated from a lambda gt10 cDNA library derived from embryonic chicken myotube cultures and was subcloned into the *Eco*RI site of pBluescript II KS(−). The *Eco*RI-*Pst*I segment of the cDNA containing the coding and 3′ untranslated TnC cDNA sequences was inserted into the corresponding sites of the expression vector pT7-7. To create mutant F22W, cDNA fragments containing the specified base changes were synthesized by polymerase chain reaction and used to replace the corresponding region of the wild-type TnC expression plasmid. The mutation was confirmed by chain-termination DNA sequencing of the entire primary region. The mutant was overexpressed in *Escherichia coli* BL21 (DE3).

The expressed mutant was isolated by a phenyl Sepharose CL B4 column in the presence of Ca^{2+} and purified using a DEAE A-25 ion exchange column. For the present studies, purified TnC F22W was dissolved in 0.1 M KCl, 25 mM 3-(*N*-morpholino)propanesulfonic acid at pH 7.2, 1 mM EGTA, and 2 mM $MgCl_2$. When Ca^{2+} was present, $CaCl_2$ was added to 2 mM. The protein concentration was approximately 0.1 mM.

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Instrumentation

For one-photon excitation at 285 nm we used the frequency-doubled output of a rhodamine 110 dye laser. The dye laser was cavity dumped at 3.976 MHz and displayed a pulse width near 7 ps. Two-photon excitation was accomplished with the fundamental output of the dye laser at 570 nm. Three-photon excitation at 855 nm was provided by a femtosecond mode-locked Tsunami Ti-sapphire laser from Spectra Physics. The repetition rate of 80 MHz was held fixed by the Loc-to-Clock accessory. The repetition rate was divided by 8 by the Lok-to-Clock electronics and used as the 10-MHz reference signal for the frequency-domain instrument. The pulse width was near 80 fs.

For multiphoton excitation the fundamental output of the dye or Ti: sapphire laser was directed to the sample compartment and focused with a laser-quality lens (2 cm focal length). Multiphoton excitation is accomplished because of the high photon density at the focal point of the lens. For intensity and anisotropy decay measurements the emission was isolated with two UG-11 filters and one WG-305 filter for all measurements. The samples were stirred during the measurements with 855-nm excitation. Without stirring the fluorescence signal with 855 nm excitation was somewhat unstable. Replacement of the illuminated volume by stirring eliminated the problem. Intensity and intensity decay measurements were performed using magic angle conditions. All measurements were performed at room temperature near 20°C, in buffer, in equilibrium with the air. Emission spectra were obtained using a ISS monochromator.

To measure the dependence of the emission on laser intensity, the peak power was attenuated with neutral density filters. To avoid any effects of widening the laser pulses by the neutral density filters, a single filter of the same design and thickness, but varying optical density, was used for the intensity measurements at various peak powers.

Frequency-domain fluorometry

The frequency-domain intensity decays of TnC were obtained on a previously described instrument (Lakowicz and Maliwal, 1985; Laczko et al., 1990) and analyzed as described previously (Lakowicz et al., 1984; Gratton et al., 1984). The intensity decay was assumed to be multiexponential,

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i}, \tag{1}$$

where α_i are the preexponential factors, τ_i are the decay times, and n is the number of exponential components. The fractional intensity of each component in the steady-state emission is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_i}. \tag{2}$$

In frequency-domain fluorometry, the sample is excited with an intensity-modulated light source, in the present case the 80-MHz output of a mode-locked Ti:sapphire laser at 855 nm or of a cavity-dumped dye laser at 285 or 570 nm. The phase angle (ϕ_ω) and the modulation (m_ω) of the emission are related to the intensity decay parameters, α_i and τ_i, and modulation frequency ω by

$$\phi_\omega = \arctan(N_\omega/D_\omega), \qquad m_\omega = (N_\omega^2 + D_\omega^2)^{1/2}, \tag{3}$$

where

$$N_\omega = \frac{1}{J}\sum_{i=1}^{n} \frac{\omega\alpha_i\tau_i^2}{1+\omega^2\tau_i^2}, \quad D_\omega = \frac{1}{J}\sum_{i=1}^{n} \frac{\alpha_i\tau_i}{1+\omega^2\tau_i^2}, \quad J = \sum_{i=1}^{n} \alpha_i\tau_i. \tag{4}$$

The values α_i and τ_i are determined by minimization of the goodness-of-fit parameter

$$\chi_R^2 = \frac{1}{\nu}\sum_{\omega,k}\left(\frac{\phi_\omega - \phi_{\omega c}}{\delta\phi}\right)^2 + \frac{1}{\nu}\sum_{\omega,k}\left(\frac{m_\omega - m_{\omega c}}{\delta m}\right)^2, \tag{5}$$

where the subscript c indicates calculated values for known values of α_i and τ_i, $\delta\phi$ and δm are the experimental uncertainties in the measured phase and modulation values, and ν is the number of degrees of freedom. For the global analysis with one- and three-photon excitation, the sum (k) in Eq. 5 extends over both sets of data.

Frequency-domain anisotropy decay data were used to recover the anisotropy decay,

$$r^k(t) = \sum_j r_{0j}^k e^{-t/\theta_j}, \tag{6}$$

where the superscript k indicates the mode of excitation (one photon ($1h\nu$), two photon ($2h\nu$), or three photon ($3h\nu$)), r_{0j}^k is the amplitude, and θ_j is the correlation time. A superscript k is not used on the θ_j values because a good fit to the data was obtained when the correlation times were assumed to be independent of the mode of excitation. The differential polarized phase and modulated anisotropy data were analyzed for each wavelength, or globally for one- and three-photon excitation, as described previously (Lakowicz et al., 1993), using a goodness-of-fit calculation similar to Eq. 5. For the global anisotropy analysis the r_{0j}^k values were allowed to vary for each mode of excitation (k), but the correlation times were assumed to be the same for all modes of excitation. For the nonglobal analysis both the r_{0j}^k values and the correlation times were variable parameters.

RESULTS

Fig. 1 shows the emission spectrum of TnC F22W excited at 855 nm. The single tryptophan residue of TnC F22W displays an unstructured emission spectrum with an emission maximum near 330 nm. These spectral properties are typical of a tryptophan residue that is partially shielded from the aqueous phase by the folded protein.

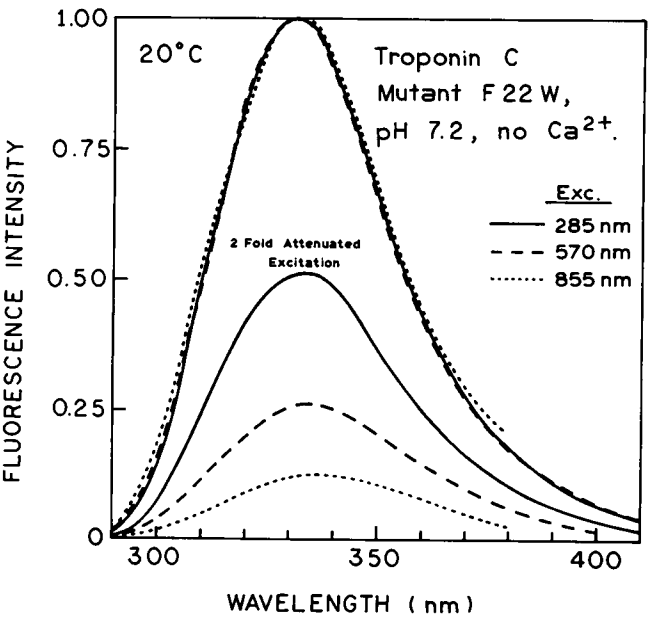

FIGURE 1 Emission spectra of Tnc mutant F22W for excitation at 285, 570, and 855 nm.

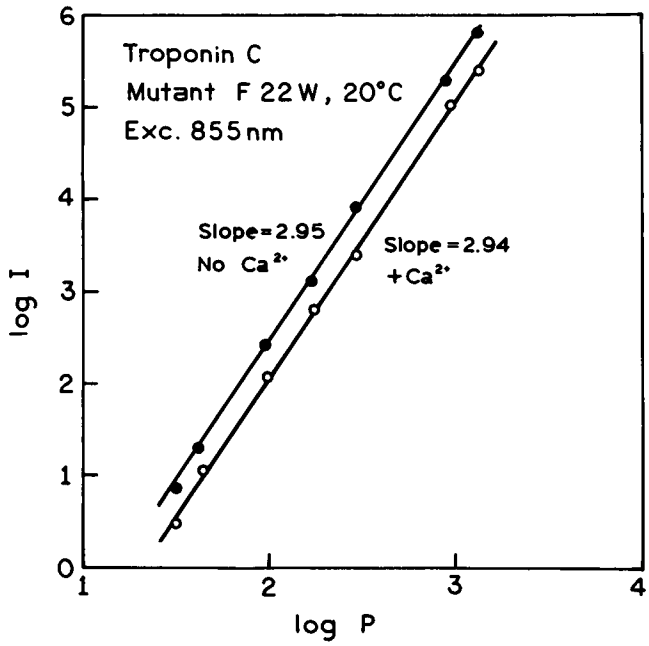

FIGURE 2 Dependence of the emission intensity of TnC mutant F22W on the incident intensity at 855 nm.

We were surprised by the ability to observe tryptophan emission at this long excitation wavelength. Two-photon excitation of TnC F22W is not expected because the one-photon absorption does not extend beyond 305, so that two-photon excitation is not expected above 710 nm. The emission spectrum observed with 855-nm excitation is essentially identical to that found with one-photon excitation at 285 nm (Fig. 1). To determine the nature of excitation at 855 nm, the intensity of the laser beam was attenuated twofold. This attenuation resulted in an eightfold decrease in emission intensity, which implies a three-photon process. At 855 nm the emission intensity of TnC F22W was found to depend almost precisely on the cube of the laser power (Fig. 2), in both the presence and absence of bound calcium.

At the shorter wavelength of 570 nm a twofold attenuation of the incident light results in a fourfold decrease in F22W intensity (Fig. 1), indicating two-photon excitation at this wavelength. This quadratic dependence on the intensity at 570 nm was found both in the presence and absence of calcium (Fig. 3).

To further characterize the emission of F22W with long-wavelength excitation we examined the intensity decays using the frequency-domain method. For excitation at 855 nm the data were measured only at integer multiples of 80 MHz. This is a consequence of the 80-MHz repetition rate of the Ti:sapphire laser. Measurements at more closely

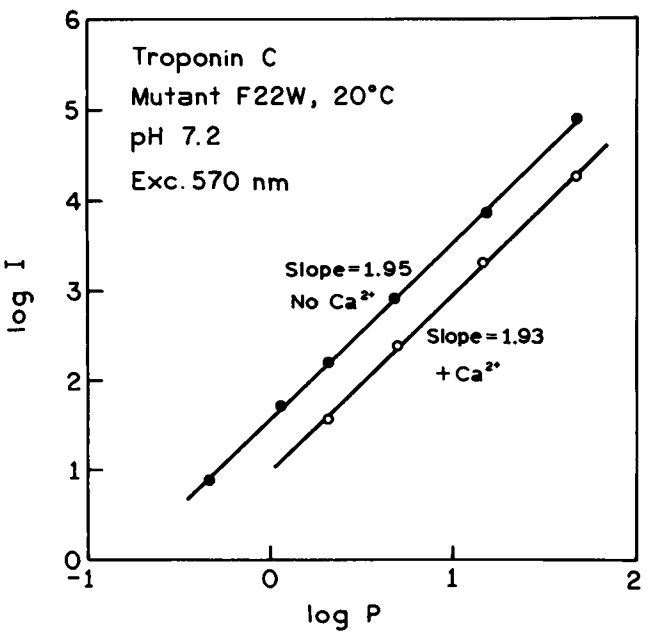

FIGURE 3 Dependence of the emission intensity of TnC mutant F22W on the incident intensity at 570 nm.

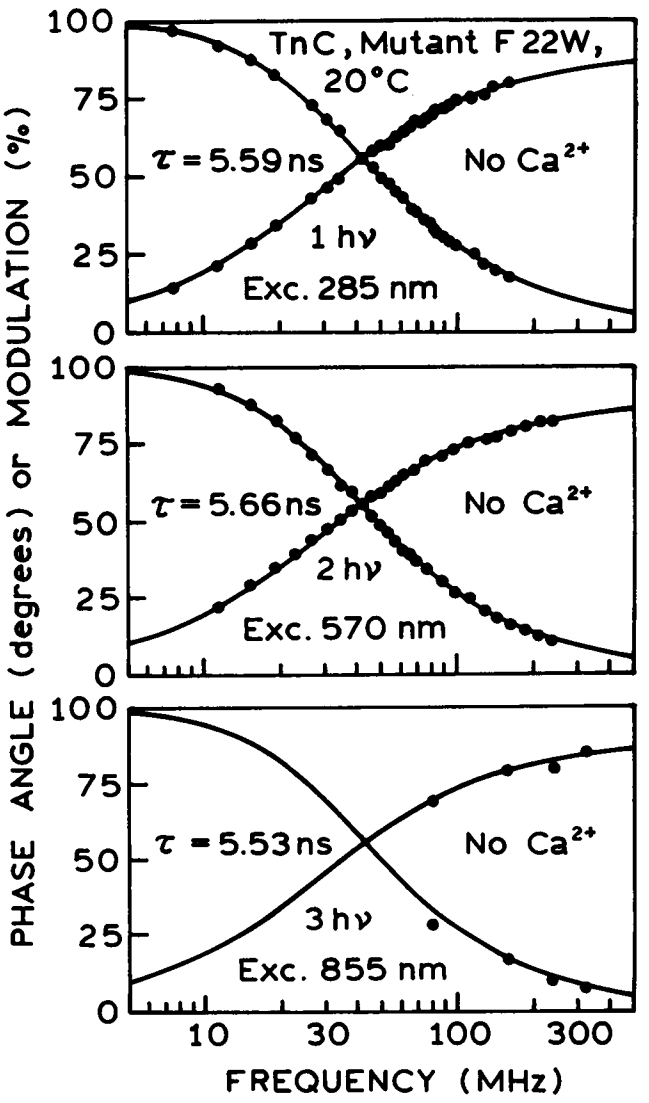

FIGURE 4 Frequency-domain intensity decays of TnC F22W without Ca^{2+} for one-, two-, and three-photon excitation.

TABLE 1 Multiexponential intensity decays of TnC mutant F22W

Conditions	Excitation mode*	τ_1 (ns)	α_1	τ_2 (ns)	α_2	$\bar{\tau}$ (ns)	χ^2_R
No Ca^{2+}	1	5.59	1.0	—	—	5.59	2.3#
	2	5.66	1.0	—	—	5.66	2.8
	3	5.53	1.0	—	—	5.53	4.9
	1–3§	5.62	1.0	—	—	5.62	2.9
$+Ca^{2+}$	1	2.00	0.089	4.56	0.911	4.46	1.5
	2	1.71	0.224	5.06	0.776	4.66	2.9
	3	1.43	0.241	5.37	0.759	5.05	4.6
	1–3§	1.24	0.121	4.70	0.879	4.57	5.4

*The excitation wavelengths were 285, 570, and 855 nm for 1hν, 2hν, and 3hν excitation, respectively.
#Calculated using $\delta\phi = 0.3°$ and $\delta m = 0.007$ as the uncertainties in the phase and modulation, respectively (Lakowicz et al., 1984; Gratton et al., 1984).
§Global analysis for one-, two-, and three-photon excitation.

spaced frequencies will be made possible in the future by a pulse picker to decrease the repetition rate of the laser. The same frequency responses were observed for excitation at 285, 570, and 855 nm (Fig. 4). The multiexponential analysis of these data is summarized in Table 1. In the absence of calcium the data were consistent by a single decay time near 5.6 ns, whereas two decay times were needed to fit the data in the presence of Ca^{2+}. However, in the presence or absence of Ca^{2+}, the decay times were unchanged for one-, two-, or three-photon excitation.

To further test the similarity of the decay times for each excitation wavelength, we performed a global analysis of the three sets of intensity decay data to a single decay law. In this analysis the values of α_i and τ_i were assumed to be the same for the three excitation wavelengths. In both the presence and absence of calcium the data for the three excitation wavelength could be fit to the same decay law (Table 1). This suggests that TnC F22W is not being perturbed by the intense illumination needed for three-photon excitation.

In contrast to the intensity decays, the frequency-domain anisotropy decays were found to be highly distinct for each excitation wavelength. Such data are shown in Fig. 5 for F22W in the absence of calcium. The positive values of the differential phase angles and modulated anisotropies are characteristic of a positive time 0 anisotropy for 285- and 570-nm excitation. The negative values indicate a negative time 0 anisotropy with 855-nm excitation. Similar data were found with bound calcium (not shown). These data for each excitation wavelength were used to recover the anisotropy decays using a nonglobal analysis (Table 2). In both the absence and presence of calcium the anisotropy decays show a short correlation time near 2 ns, which accounts for about half of the total anisotropy. The crystal structure of apo-TnC shows that the native residue Phe22 is partially buried and appears to have a restricted motional freedom (Herzberg et al., 1986). Molecular graphics suggest a similarly buried Trp22 in the apo mutated structure and a slightly more exposed Trp22 in the Ca^{2+}-bound state. These structural features are borne out by the high quantum yields of Trp22 in both the apo state (0.33) and Ca^{2+}-bound state (0.25) (She et al., 1996) and are consistent with the short correlation time of about 2 ns. The anisotropy decays also display a longer correlation time near 11 ns, which is consistent with the overall rotational diffusion of the protein.

Examination of Table 2 reveals that for three-photon excitation the long correlation time is unreasonably high (72 ns). However, this value is less certain for 855-nm excitation because the total anisotropy amplitude is small, and the high correlation time may be the result of the limited information in the data for three-photon excitation. Overall, the correlation times are roughly similar for 1hν, 2hν, and 3hν excitation. In contrast to the correlation times, the anisotropy amplitudes are strongly dependent on the mode of excitation. The time 0 anisotropy ($r_0^k = \Sigma_j r_{0j}$) decreases for 2hν excitation and becomes negative for 3hν excitation (Table 2).

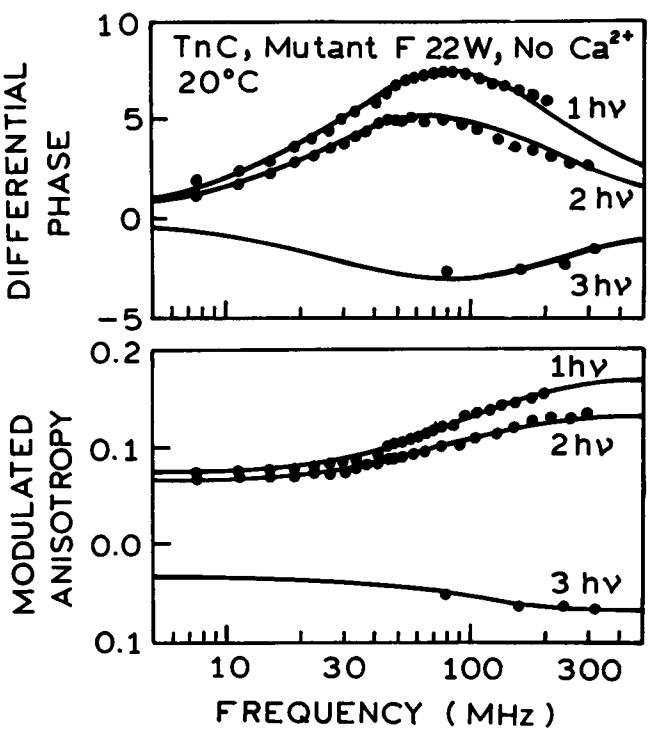

FIGURE 5 Frequency-domain anisotropy decays of TnC F22W for one-, two-, and three-photon excitation.

TABLE 2 Anisotropy decay of TnC mutant F22W

Conditions	Excitation mode	θ_1 (ns)	r^k_{01}	θ_2 (ns)	r^k_{02}	r^k_0	χ^2_R
No Ca^{2+}	1*	1.75	0.104	7.56	0.079	0.183	0.9#
	2	2.89	0.084	18.0	0.049	0.133	1.2
	3	2.14	−0.043	72.0	−0.026	−0.069	0.7
	1–3§	1.97	0.117	9.35	0.064	0.181	1.0
$+Ca^{2+}$	1	1.76	0.097	7.45	0.088	0.185	0.8
	2	1.91	0.091	44.4	0.033	0.124	0.9
	3	1.63	−0.036	28.2	−0.023	0.059	0.9
	1–3§	1.90	0.120	11.9	0.066	0.186	1.1
			0.078		0.044	0.122	
			−0.035		−0.022	−0.057	

*See Table 1.
#The uncertainties were $\delta\Delta = 0.3°$ and $\Delta\Lambda = 0.007$ for the differential phase and modulation ratio, respectively (Lakowicz et al., 1993).
§The correlation times θ_j were global for all excitation wavelengths. The amplitudes (r_{0i}) were nonglobal.

To determine the similarity of the correlation times we performed a global analysis. Because the time 0 anisotropies were dependent on excitation wavelength, these values were nonglobal in the analysis. In the global analysis the correlation times were assumed to be the same for one-, two-, and three-photon excitation. We obtained satisfactory fits when the same correlation times were used for all excitation wavelengths (Table 2). Furthermore, roughly the same fraction of the total anisotropy decayed by the fast (2 ns) and slower (9–11 ns) motions for each excitation wavelength. Because the anisotropy decays of proteins are known to be highly sensitive to the three-dimensional structure, the similar anisotropy decays with 1hν, 2hν, and 3hν excitation indicate that 3hν excitation of this single tryptophan protein can be accomplished without perturbation of its structure.

We were surprised by the negative time 0 anisotropy observed for 3hν excitation. The excitation anisotropy spectra of F22W were examined over the range of wavelengths available with our lasers. These anisotropy spectra show that 2hν excitation from 560 to 600 nm shows anisotropy values lower than 1hν excitation (Fig. 6). For 3hν excitation the steady-state anisotropy is negative from 830 to 900 nm excitation.

DISCUSSION

What is the origin of the negative anisotropy of the tryptophan residue in TnC F22W with three-photon excitation? It is well known that indole and tryptophan display complex excitation anisotropy spectra with ultraviolet excitation (Weber, 1960; Konev, 1967). This behavior is thought to be due to spectral overlap of two electronic transitions ^1L_a and ^1L_b, whose transition moments are perpendicular to each other (Valeur and Weber, 1977; Eftink et al., 1990). With the exception of 5-methyl indole (Eftink and Chen, 1994), the emission from tryptophan derivatives is due predominantly to the ^1L_a state. The negative anisotropy implies absorption into the ^1L_b state, followed by emission from the perpendicular transition of the ^1L_a state. In the case of two-photon excitation Rehms and Callis have already shown that both the ^1L_a and ^1L_b states contribute to the two-photon absorption spectrum of indole (Rehms and Callis, 1993). Hence the data for F22W suggest that 3hν excitation is due to absorption of the ^1L_b state, which seems to be the dominant transition with three-photon excitation. However, we cannot exclude the possibility that the ^1L_a and ^1L_b states display different polarizations for one-, two-, and three-photon excitation. The possibility of one-, two-, and three-photon excitation of proteins should provide additional information on the validity of the ^1L_a-^1L_b model for tryptophan fluorescence.

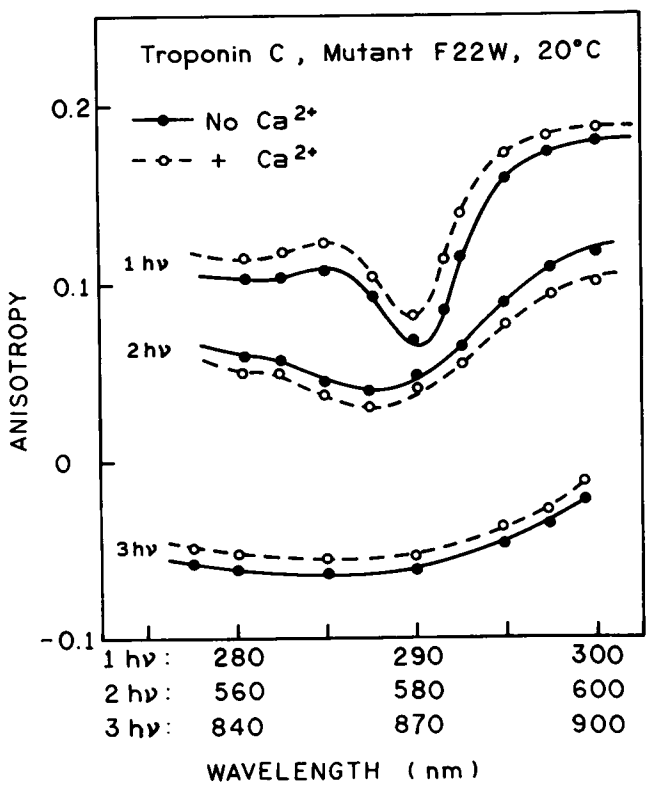

FIGURE 6 Excitation anisotropy spectrum of TnC mutant F22W for one-, two-, and three-photon excitation.

A useful conclusion from this work is that three-photon excitation of protein can be accomplished using the fundamental output of a Ti:sapphire laser. Such lasers are now in use for two-photon excitation in fluorescence microscopy (Denk et al., 1990, 1994, 1995; Lindek et al., 1995). Hence one can imagine the use of intrinsic protein fluorescence in microscopy, which heretofore has been inaccessible because of the lack of suitable optics for one-photon excitation.

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