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Biophysical Chemistry 79 (1999) 25–32

Biophysical Chemistry

Three-photon excitation of *N*-acetyl-L-tyrosinamide

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Received 10 August 1998; received in revised form 25 February 1999; accepted 25 February 1999

Abstract

We observed emission from the tyrosine derivative *N*-acetyl-L-tyrosinamide (NATyrA) when excited with the fundamental output of a femtosecond Ti:Sapphire laser from 780 to 855 nm. The dependence on incident laser power indicates a three-photon process. The emission spectra and intensity decay in glycerol–water (30:70) at 5°C were found to be identical for one- and three-photon excitation. Also the excitation spectrum of three-photon-induced fluorescence of NATyrA corresponds to the one-photon excitation spectrum. The time-zero or fundamental anisotropy spectrum was reconstructed from the frequency-domain anisotropy decays. The three-photon anisotropies are similar or larger than the one-photon anisotropies. These three-photon anisotropies are surprising given the near zero values known for tyrosine with two-photon excitation. The observations indicate that one- and three-photon excitation directly populates the same singlet excited states(s). However, the origin of the anisotropies with multi-photon excitation of tyrosine remain unclear and unpredictable.

1. Introduction

Two- and three-photon excitation of fluorescence have recently been used in spectroscopic studies of biomolecules [1–4] and in microscopic studies of cellular imaging [5–8]. In fluorescence microscopy, multi-photon excitation offers conditions equivalent to confocal microscopy without the complexity associated with confocal systems. It has already been shown that three-photon excitation provides significantly better localization of excitation under microscopic [9,10] or spectroscopic [11,12] conditions. Two- and three-photon excitation is now being extended to imaging of chromosomes and even tissue samples [13–15].

While initial biochemical studies of multi-photon excitation (MPE) used extrinsic fluorophores, there is considerable interest in using MPE with the intrinsic fluorophores in proteins [8,16]. There have been several experimental and theoretical reports on two-photon excitation of indoles

Abbreviations: NATyrA, *N*-acetyl-L-tyrosinamide; FD, frequency-domain; MPE, multi-photon excitation

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0301-4622/99/$ - see front matter
PII: S0301-4622(99)00033-2

[17–19] and tyrosine [20,21]. These studies resulted in surprising observations. The two-photon induced fluorescence anisotropies of tryptophan were found to be lower values than observed for one-photon excitation, whereas indole anisotropies are higher for two-photon excitation than for one-photon [19,22]. In addition, three-photon induced anisotropies of tryptophan have negative values for tryptophan and proteins [23,24]. The anisotropy spectrum for tyrosine measured with two-photon excitation showed unexpectedly low values near zero for relatively wide range of excitation wavelengths [21,25].

To date, all emission spectra have been the same for one-photon and multi-photon excitation of indole and phenols. However, the anisotropies have been unpredictable. We believe that systematic collection of experimental data will stimulate theoretical studies on the multi-photon spectral properties of the aromatic amino acids, and eventually to an understanding of their excitation anisotropy spectra. In previous reports we described the spectral properties of tyrosine and tyrosine proteins with two-photon excitation [21,25]. In the present report we extend these studies to three-photon excitation of *N*-acetyl-L-tyrosinamide.

2. Materials and methods

N-acetyl-L-tyrosinamide (NATyrA) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) and was used without further purification. NATyrA was dissolved in glycerol–water (30:70 v/v) at 7×10^{-3} M. The solution was in equilibrium with air. Frequency-domain intensity and anisotropy decays were obtained using instrumentation described previously [26,27].

Three-photon excitation 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 eight by the Loc-to-Clock electronics, and used as the 10-MHz reference signal for the FD instrument. The pulse width was near 90 fs.

The fundamental output of the Ti:Sapphire was brought directly to the sample compartment and focused with a laser-quality lens (2-cm focal length). For all time-resolved measurements the emission was isolated with two UG-11 filters and one WG-290 filter. The samples were stirred during the measurements. Intensity and intensity-decay measurements were performed using magic angle conditions.

For one-photon excitation we used third harmonic generated by frequency tripler (INRAD, Model 5–050). For emission spectra with 840-nm excitation we used an ISA monochromator (8-mm bandpass) with one UG-11 emission filter, and corrected the spectra for the transmission of the UG-11 filter. The signals from the solvents alone were less than 2% of that observed in the absence of NATyrA. For measurements of 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. All measurements were performed at 5°C.

The FD intensity decays of NATA were analyzed as described previously [26,27]. In FD fluorometry, the sample is excited with an intensity-modulated light source, in the present case the output of a mode-locked Ti:Sapphire laser. The intensity decay was assumed to be multi-exponential:

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

where α_i are the pre-exponential factors, τ_i are the decay times, and n the number of exponential components. 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:

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

where:

$$N_\omega = \frac{1}{J}\sum_{i=1}^{n}\frac{\omega\alpha_i\tau_i^2}{1+\omega^2\tau_i^2}, \quad D_\omega\frac{1}{J}\sum_{i=1}^{n}\frac{\alpha_i\tau_i}{1+\omega^2\tau_i^2},$$
$$J = \sum_{i=1}^{n}\alpha_i\tau_i. \tag{3}$$

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

$$\chi_R^2 = \frac{1}{\nu}\sum_{\omega}\left(\frac{\phi_\omega - \phi_{\omega c}}{\delta\phi}\right)^2 + \frac{1}{\nu}\sum_{\omega}\left(\frac{m_\omega - m_{\omega c}}{\delta m}\right)^2, \tag{4}$$

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 one-photon excitation: $\delta\phi = 0.2°$ and $\delta m = 0.005$; for three-photon excitation: $\delta\phi = 0.4°$ and $\delta m = 0.01$. Larger values were used for the standard errors in three-photon excitation to account for the lower signal-to-noise ratio in these measurements.

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

$$r(t,\lambda) = r_{0k}(\lambda)\mathrm{e}^{-t/\theta} \tag{5}$$

where r_{0k} is the time-zero anisotropy for 1 $h\nu$ or 3 $h\nu$ excitation (k), and θ is the rotational correlation time. The differential polarized phase and modulated anisotropy data were analyzed simultaneously for five excitation wavelengths. The correlation time θ was assumed to be a global parameter and the anisotropy amplitudes $r_{0k}(\lambda)$ were dependent on excitation wavelength λ, as described previously [28,29]. The global analyses were performed separately for one- and three-photon excitation. This was done to reconstruct the excitation anisotropy spectrum from the time-zero anisotropies. Of course, the global analysis may also include both one- and three-photon data as we have shown previously [13].

It is difficult to measure absorption or excitation spectra with two-photon excitation, and at present there are no three-photon standards. We measured the three-photon excitation spectra of NATyrA by measuring the emission intensity divided by the cube of the average incident power as measured with a power meter. The autocorrelation pulse-width at 800 nm was 90 fs as estimated by Dr Gary Eisenman from Coherent. The spectral width of the pulses was 12 nm and was adjusted to this value for all wavelengths. We did not measure the autocorrelation pulse-widths for all wavelengths. However, from our experience we know that the pulse-width does not change more than 20% within spectral region used in this experiment (780–855 nm). In particular we have performed the following test. We measured the two-photon induced fluorescence signals for fluorescein at several excitation wavelengths between 780 and 840 nm. The plot of these fluorescence signals divided by square of laser power (I_F/P^2) agreed well with two-photon absolute cross-sections measured for fluorescein [30]. We obtained similar agreement within 20% for rhodamine B. Any substantial change in pulse-width should result in disagreement of our plots and the reported corrected cross-sections.

3. Results

3.1. Selection of solvent

Excitation anisotropy spectra are usually measured in high viscosity solutions when the rotational correlation time is much longer than the fluorescence lifetime. However, we were unable to measure the excitation anisotropy spectrum of NATyrA in 100% glycerol at −60°C. The three-photon cross section for NATyrA appears to be low, approximately 10-fold lower than that of the *N*-acetyl-L-tryptophamide (NATA) under comparable conditions. Also, NATyrA had a modest solubility limit in glycerol. These factors resulted in emission signals from NATyrA which were low and unstable using the fundamental output of the Ti:Sapphire laser as the excitation source. Hence we decided to reconstruct the excitation anisotropy spectrum from the time-resolved anisotropy decays in a less viscous solvent.

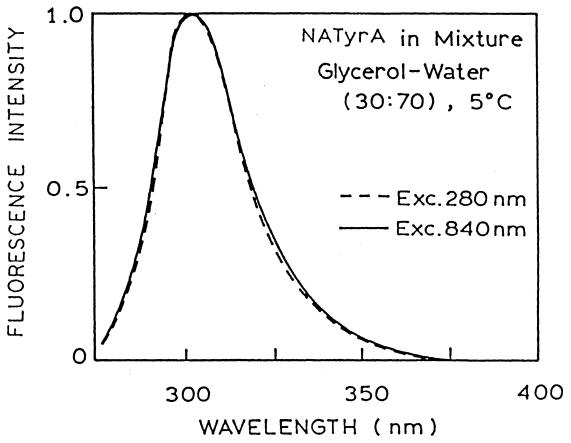

Fig. 1. Fluorescence spectra of NATyrA in a glycerol–water (30:70) mixture at 5°C for excitation at 280 nm (one-photon) and 840 nm (three-photon).

3.2. Fluorescence spectra and lifetime

As a less viscous solvent we chose a glycerol–water mixture (30:70, v/v) at 5°C. Under these conditions the correlation time is closer to the lifetime. Emission spectra of NATyrA are shown in Fig. 1 for excitation at 280 and 840 nm. The emission spectra are essentially identical, indicating that the emission arises from the same lowest singlet state with 280 and 840 nm excitation. Since NATyrA does not absorb above 300 nm, it seemed unlikely that two-photon excitation could occur with wavelengths above 600 nm. To determine the mode of excitation at 840 nm we examined the dependence of the emission intensity on laser power (Fig. 2). We found the dependence characteristic of three-photon excitation. Similar results were obtained from 780 to 855 nm (not shown).

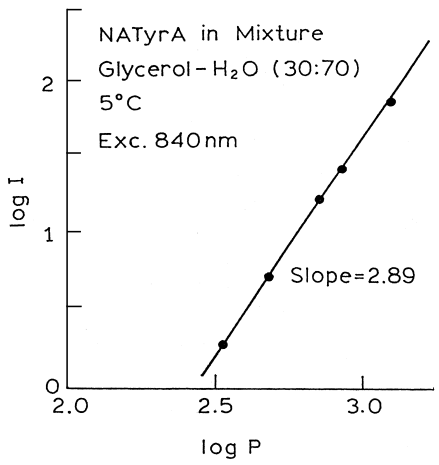

Fig. 2. Dependence on laser power (p) of the emission intensity (I) of NATyrA.

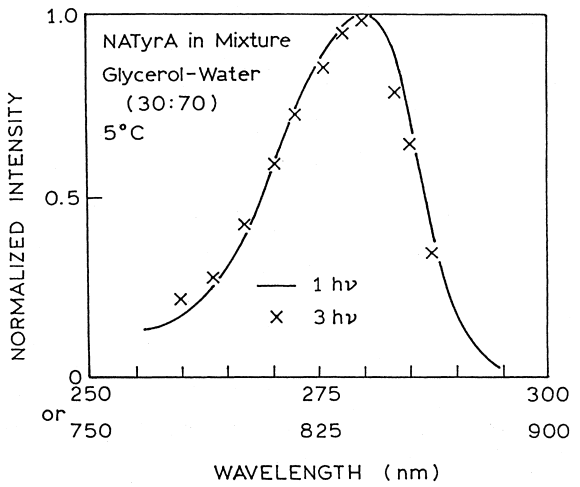

Fig. 3. Fluorescence excitation spectra for one-photon (—) and three-photon absorption. To determine the three-photon excitation, the fluorescence signal (I) was divided by the cube of the laser power (P) and then normalized.

It is known that the absorption spectra can be different for one- and two-photon excitation. To be more precise, the two-photon absorption spectrum need not look like the one-photon spectrum shifted to twice the wavelength. Hence we determined the three-photon excitation spectrum of NATyrA (Fig. 3). The results indicate that the excitation spectra are essentially identical. We note that it is difficult to accurately measure multi-photon excitation spectra, so that small changes in the spectral shape may be present but

Table 1
Intensity decays of *N*-acetyl-L-tyrosinamide with one and three-photon excitation

Excitation	τ_1 (ns)	τ_2 (ns)	α_1	α_2	χ_R^2
1 $h\nu$, 280 nm	0.53	2.87	0.086	0.914	0.8 (14.1)[a]
3 $h\nu$, 840 nm	0.92	2.63	0.011	0.989	0.9 (4.2)

[a] In parentheses are the χ_R^2 values for single exponential fits.

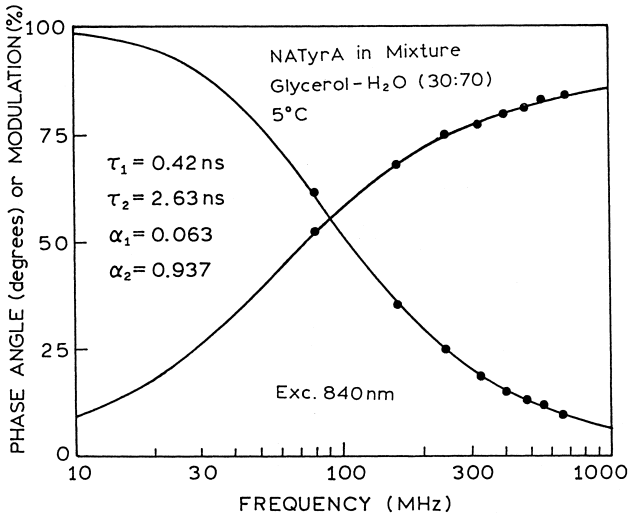

Fig. 4. Frequency-domain intensity decay of NATyrA in glycerol–water (30:70) mixture at 5°C with three-photon excitation.

not observable with the present degree of precision.

We also examined the intensity decay of NATyrA with one- and three-photon excitation (Fig. 4). Similar intensity decays was observed for one-photon and three-photon excitation (Table 1). Once again this indicates the same excited state for one- and three-photon induced fluorescence. The intensity decays do not depend on excitation wavelength. The same intensity decays were observed for 780 nm excitation, 840 nm or for 855 nm.

3.3. Excitation anisotropy spectrum with three-photon excitation

We measured anisotropy decays of NATyrA in glycerol–water mixture for five excitation wavelength from 780 nm to 855 nm. These frequency-domain data were fitted simultaneously to yield one global correlation time θ and five non-global zero-time anisotropies r_{03} (λ) (Fig. 5). The goodness of fit χ_R^2 was 0.98 and did not improve for the two correlation time model. The recovered correlation time of 251 ± 4 ps is approximately fourfold longer than in aqueous solution [31] due to the higher viscosity. The zero-time anisotropies are dependent on excitation wavelength and ranged from 0.256 for 855 nm to 0.119 for 780

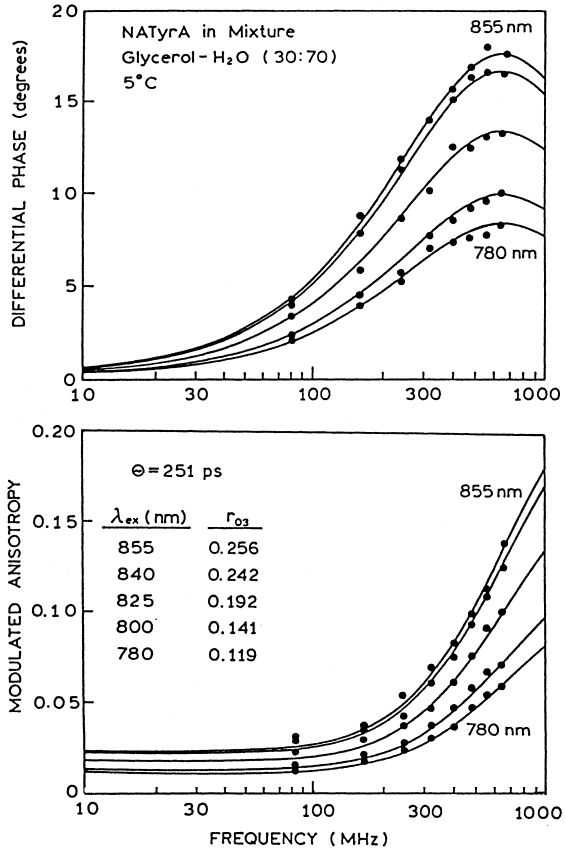

Fig. 5. Frequency-domain anisotropy decays of NATyrA for three-photon excitation from 780 to 855 nm. The lines are from global fit to the data (●).

nm. The standard deviations for $r_{03}(\lambda)$ were lower than 0.003 for all wavelengths. Hence, the zero-time anisotropies can be recovered from time-resolved data with high confidence. The wavelength dependent zero-time anisotropies represent the excitation anisotropy spectrum (Fig. 6).

The anisotropy spectrum of NATyrA with three-photon excitation is similar to that found with one-photon excitation (Fig. 6). Three-photon excitation results in anisotropy values 1.65-fold larger due to $\cos^6\theta$ photoselection [32]. Following this correction our results indicate that the fundamental anisotropy of NATyrA for three-photon excitation is approximately 60% of that for one-photon excitation. This can be seen by the three-photon values corrected to one-photon photoselection (Fig. 7).

4. Discussion

Prior to discussing the results it is valuable to question how well the time-resolved data recover the actual time-zero anisotropies. Comparison of the three-photon excitation anisotropy spectrum (Fig. 6, solid circle) with low temperature spectrum of NATyrA in glycerol (Fig. 6, dashed line) shows that the shape is similar, but the anisotropies recovered from time-resolved data for three-photon excitation are lower. A similar difference was observed for the time-zero anisotropies observed with one-photon excitation. Hence, a small fraction of the total anisotropy may be lost due to unresolved fast motions, but this effect is similar for both the one-photon and three-photon data.

Since the measured anisotropy values are similar for one- and three-photon excitation we considered the possibility of the presence of the third harmonic when illuminated at 780–855 nm. We could not detect any signal at the third harmonic wavelength from either the solvent or the NATyrA solution itself.

In summary, our results indicate that the three-photon anisotropy of NATyrA is very similar to the one-photon values. When corrected for $\cos^2\theta$ vs. $\cos^6\theta$ photoselection, the three-photon values are approximately 65% of the one-photon anisotropies. Further theoretical studies are needed to explain these results.

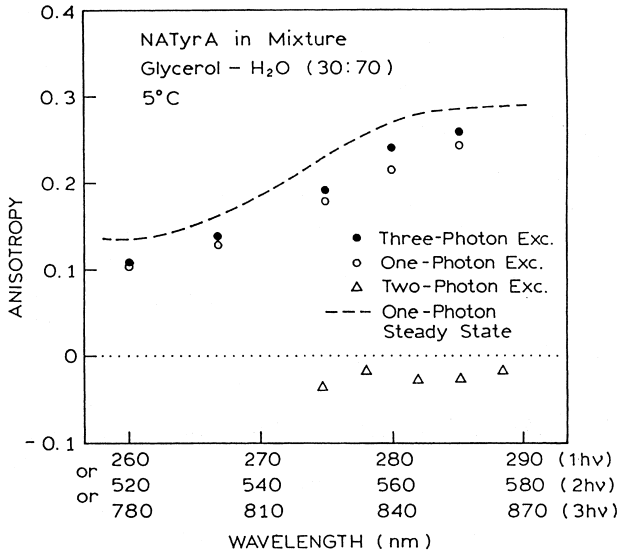

Fig. 6. Excitation anisotropy spectrum for three-photon induced fluorescence of NATyrA (●) reconstructed from time-resolved measurements. Also shown are one-photon values from time-resolved (○) and steady state (- - -) data. The values near zero (Δ) are the fundamental anisotropies observed for two-photon excitation. Steady state measurements were performed in glycerol at −60°C using SLM 800 spectrofluorometers.

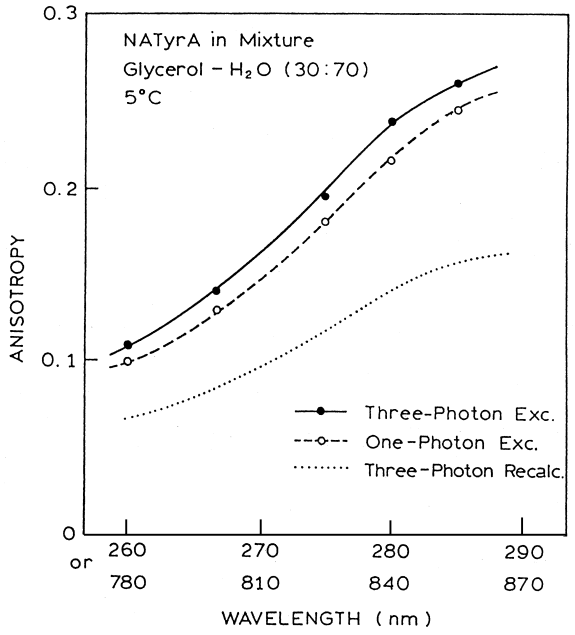

Fig. 7. Comparison of the one, two and three-photon excitation anisotropy spectra of NATyrA. Also shown are the three-photon anisotropies corrected from $\cos^6\theta$ to $\cos^2\theta$ photoselection.

Acknowledgements

This work was supported by a grant from the National Center for Research Resources, RR-08119.

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