Visible Emission of a Photoproduct from Tryptophan Solution Induced by Multiphoton Excitation: An Investigation by Intensity Fluctuation Analysis

Goro Nishimura* and Masataka Kinjo

Research Institute for Electronic Science, Hokkaido University, N12W6, Sapporo 060-0812, Japan

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Strong visible emission from a tryptophan methylester solution was observed by a confocal microscope with a 795-nm femtosecond pulsed laser. The emission was presumably similar to that observed in 5-hydroxytryptamine (serotonin) solution. In contrast to the intensity in 5-hydroxytryptamine solution, the intensity was proportional to the seventh power of the excitation intensity. The fluctuation analysis of the intensity, which was used to characterize the emission, showed that the concentration of the species of the emission was proportional to the fifth power of the excitation intensity, and its brightness, to the second power. Thus, the emission is attributed to the emission from the two-photon excited state of a photoproduct generated by the five-photon process. The decay time of the correlation function was significantly shortened with an increase in the excitation power. This suggests that the photoproduct converts a nonradiative species, such as a nonfluorescent product, from its excited state generated by the two-photon process. The fluctuation analysis of the novel emission of tryptophan can be applied to tryptophan residues in proteins.

Introduction

Single-molecule detection (SMD) is recognized as a powerful tool in the biochemical field. Fluorescence detection is the most common technique in SMD, and fluorescence correlation spectroscopy is one of the most interesting applications.[1] The fluorescence labeling of samples by means of chemical or protein engineering is a key issue in SMD. SMD in the UV region, however, is expected to solve the labeling-procedure problem in many cases. For instance, it will be applicable to the analysis of enzyme kinetics because aromatic amino acids such as tryptophan and tyrosine are contained in many kinds of proteins. The problems of SMD in the UV region are the optics and the background. The multiphoton excitation technique is considered to be a solution to these problems because the longer excitation light reduces those of the optical components and the background due to light scattering and unfavorable emission from the optics.

Multiphoton excitation, especially two-photon excitation, is a common technique in biological imaging, and the excitability of a wide range of dyes was examined.[2,3] An application of three-photon excitation to the intrinsic UV dyes was first demonstrated, and the imaging of 5-hydroxytryptamine (5HT; serotonin) in cells was investigated.[4] A detailed analysis of the three-photon emission of tryptophan derivatives was also reported.[5] This technique is presumably extended to measurements in the SMD level of tryptophans. However, it was not applied because of problems with the emission efficiency, optics in UV, and photostability. The measurement of the UV fluorescence of tryptophan excited by two photons has recently been demonstrated with a special protein, hemocyanin containing 148 tryptophan residues.[6]

The visible (vis) emission of 5HT with multiphoton excitation was first reported in 1997.[7] The similar emission of tryptophan, which is 10 to 100 times weaker than the emission of 5HT, was briefly described above. The novel emission of 5HT solutions was analyzed by the transient excitation method by using a submillisecond optical gating technique, and it was concluded that the emission was attributed to the emission of the photoproduct generated through the four-photon excitation process of 5HT. However, there was no further analysis of the vis emission of 5HT and tryptophan with the exception of the application to the highly sensitive detection of 5HT in capillary electrophoresis.[8−10]

In this report, we investigated the vis emission of a tryptophan methylester solution and found that the emission intensity varied according to the seventh power of the excitation intensity. The vis emission was analyzed by the correlation method, which identifies the number and brightness of the vis emitting species in the specific volume defined by the optics to investigate the mechanism of the emission. We have concluded that a photoproduct of tryptophan is generated through the five-photon process, in contrast to that in 5HT. The decay rate of the correlation function and the photostability of the product are discussed.

Materials and Methods

L-Tryptophan methylester hydrochloride (TrpMe; Nacalai tesque, Kyoto, Japan) and 5-hydroxytryptamine hydrochloride (5HT, serotonin; Sigma, St. Louis, MO), which were purchased commercially, were dissolved in 10 mM Tris·HCl at pH 7.4 without further purification. The solution of about 100 μL was dropped onto an eight-chambered coverglass (Lab-Tek, Nalge Nunc, Naperville, IL), and its measurements were carried out at room temperature. The concentration change due to evaporation was negligible during the measurements.

A rhodamine 123 (R123; Sigma, St. Louis, MO) aqueous solution was used for the calibration of the optics and the standard curve of the fluorescence correlation function.

An inverted epifluorescence microscope (IX-70, Olympus, Tokyo, Japan) was modified to lead the pulsed laser light and

* To whom correspondence should be addressed. E-mail: gnishi@imd.es.hokudai.ac.jp.

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detect the UV emission. A new laser-light port was made at the cube filter turret. A special filter cube with a near-infrared/visible dichroic mirror, about 100% reflection at 750−850 nm and about 80 to 90% transmission in the range from 300 to 700 nm, guided the laser light to the objective lens. The tube lens for detection was replaced by a quartz lens to improve the UV transmission. A pinhole (100 μm in diameter) was located at the image plane and band-pass filters (320−390-nm band-pass for UV detection (340-nm filter) and 410−540-nm band-pass for the vis detection (490-nm filter)) were inserted between the pinhole and a photomultiplier tube module (H5783P-03 or H7421-40, Hamamatsu Photonics, Hamamatsu, Japan). The sensitivity of the H7421-40 module was 3 to 5 times better than that of H5783P-03 in the vis range, though it was about half that of H5783P-03 in the UV range. The experimental results were essentially the same with each detector. The position of the pinhole could be adjusted by *x*, *y*, and *z* stages to obtain the maximum detection intensity.

The optimum pinhole position for detection of the vis emission was adjusted with R123 solution to maximize the intensity. However, the position was adjusted with the UV emission of a TrpMe solution at 80-mW excitation for the alignment of the UV detection because the axial position was different from that of the vis emission because of the dispersion of the lens.

A Ti:sapphire laser with a standard mirror set (Tsunami, Spectra Physics, Mountain View, CA) pumped by the second harmonic of a Nd^{3+}:YAG laser (Millennium X, Spectra Physics, Mountain View, CA) excited the sample at 795 nm. The pulse width and the power of the laser were about 80−100 fs and about 1600 mW, respectively. The laser was fed into a compensator (Mira SPO, Coherent, Palo Alto, CA) and then expanded to fill the back aperture of the objective lens (UApo/340 40x, NA 1.15 water, Olympus, Japan). The beam diameter at the objective lens, about 5 mm, was slightly smaller than the aperture diameter of the lens. The laser power could be changed by a variable neutral density (ND) filter (VND100, Sigma Koki, Hidaka, Japan) that was slightly tilted from its beam axis to avoid any back reflection. The power was monitored between the ND filter and the beam expander by a power meter (model 200, Coherent, Palo Alto, CA) or a silicon detector power meter (TQ8215/TQ82010, Advantest, Tokyo, Japan). The power at the objective lens was about 60% of the measured power. The value of the power in the following results is shown by the measured value. The power density values in the discussion are shown by the calibrated value with the efficiency.

The electronic pulses of the photomultiplier tube (PMT) module, H5783P-03, were amplified by a high-speed amplifier (WP-33, Kuranishi, Tokyo, Japan) and discriminated by a constant fraction discriminator (TC454, Tennelec, Oak Ridge, TN). Then, they were converted to transistor−transistor logic (TTL) pulses by a home-built discriminator using a comparator integrated circuit (AD9687BD, Analogue Devices, MA) and fed into a digital correlator board (ALV5000/E, ALV GmbH, Langen, Germany). Using H7421, the module consisted of an optimum amplifier and a discriminator, and eventually the electronic pulse could be fed directly into the correlator board. The correlation function and the counting-rate history were analyzed by software (gnuplot v 3.71) on another computer. The data were accumulated in 5 min to obtain the correlation functions.

The standard measurement of the fluorescence correlation function with R123 yielded 0.062 ms at 14.4-mW excitation, which was the maximum intensity needed to maintain the square dependence on the excitation power below the saturation of the emission. The values varied about 15% from day to day. The diffusion time yielded the beam waist of the optics at about 0.27 μm in the e^{-2} radius. The structure parameter, which is the ratio of the e^{-2} length of the *z* axis and its radius in the *xy* plane, was about 6.7. Therefore, the volume element, which is the effective excitation-detection volume, could be estimated to be about 0.75 fL. The average power density was also estimated to be 7.6×10^6 W/cm^2 at the center of the Gaussian beam, and the instantaneous peak power density was about 1.2×10^{12} W/cm^2. The parameters obtained with R123 were regarded as the standard in the analysis.

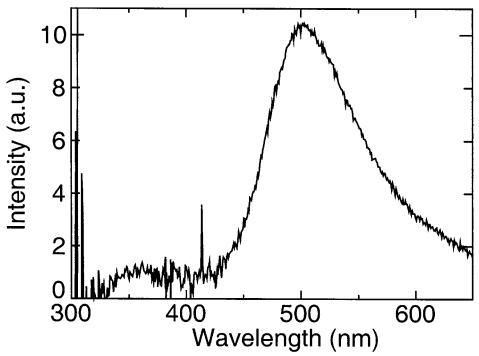

Figure 1. Emission spectrum from 4 mM TrpMe in 10 mM Tris·HCl buffer, pH 7.4, excited by a 795-nm femtosecond pulse laser at 200 mW. The background of the buffer solution was subtracted. The emission spectrum did not change with various excitation intensities from 100 to 200 mW.

The emission spectrum of the solution was carried out by replacing the PMT by an optical fiber and measured by a photonic multichannel analyzer (C7473-36, PMA-11, Hamamatsu Photonics, Hamamatsu, Japan). The pinhole at the image plane was removed in the measurements. The fiber transmittance and detector sensitivity were corrected by the manufacturer. The transmittance spectra of the objective, the dichroic mirror, and the emission filter were measured. Then, the measured spectrum was calibrated by the spectra. The absolute value of the transmittance of the objective was not calibrated. The vis emission spectrum was measured with an infrared cut filter, and the UV emission spectrum was measured with the 340-nm filter. The background that was measured with the buffer solution was subtracted.

Results and Discussion

The multiphoton excitation emission of the TrpMe solution is shown in Figure 1. The emission spectrum shows two peaks at about 360 and 503 nm. The peak at about 360 nm is the fluorescence of TrpMe. Another peak at 503 nm was not observed with one-photon excitation. The emission peak of the emission spectrum at 503 nm did not change with different excitation powers (100−200 mW) at 795 nm. The emission intensity of the vis region (400−650 nm) was strongly dependent on the excitation power. This vis emission was not observed without TrpMe at a lower excitation power below about 160 mW (peak power density 1.3×10^{13} W/cm^2). A weak vis emission, which could not be detected by the spectrometer, was observed by the PMT at more than 160 mW with the Tris·HCl buffer solution. This vis emission was considered to originate from the laser-induced breakdown (LIB) and to be the white continuum of water. There are known to occur at more than 10^{13} W/cm^2 because of the high-order nonlinear effect with the high electric field.[11] Under our experimental conditions, this

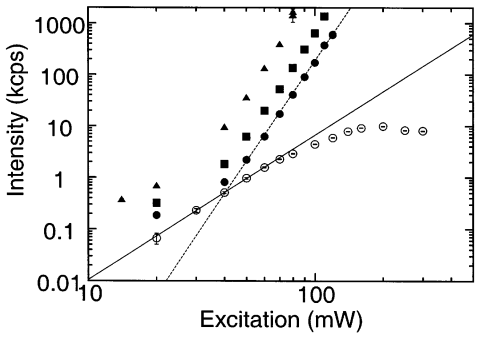

Figure 2. Emission intensity of TrpMe in 10 mM Tris·HCl buffer, pH 7.4, as a function of excitation intensity. Visible emissions within the 410−490 nm range of (●) 0.1, (■) 1, and (▲) 4 mM TrpMe solutions. (○) UV emission within the 320−390 nm range of a 0.1 mM TrpMe solution. Fitting results for (—) $I_{exc}^{2.8}$ and (- - -) $I_{exc}^{6.6}$.

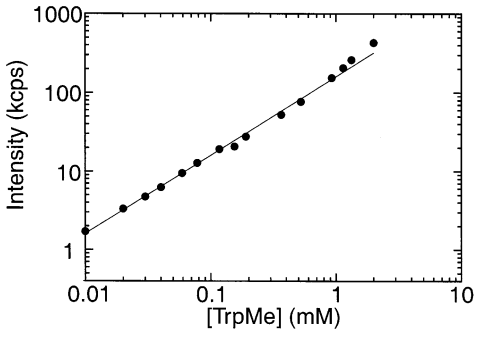

Figure 3. Intensity of vis emission from the TrpMe solution with 80-mW excitation. The background intensity, about 0.8 kcps, was subtracted. It is proportional to the concentration of TrpMe (—).

emission of the solvent was negligible. With the exception of the emission intensity, the vis emission spectrum in a 5HT solution had a peak of 503 nm and was essentially the same as that in the TrpMe solution. Therefore, the similar origin of the vis emission of the TrpMe solution was expected.

The emission spectrum with a lower concentration did not change, although a new emission shoulder around 550−600 nm was observed with an increase in the concentration above ∼10 mM. Other photodynamic processes might have contributed to the vis emission at higher concentrations. The following measurements were carried out under a concentration lower than 10 mM.

The photon-counting rate of the vis emission from TrpMe was almost constant with the exception of the fluctuation of the excitation laser during long illuminations of about 5 h. A steady state was maintained under our experimental conditions.

A similar emission was observed with the L-tryptophan solution. The excitation power dependence and other characteristics of the vis emission, which are discussed later, were essentially the same. The possibility of contamination could be excluded because the vis emission with tryptophan purchased from various companies and with other tryptophan derivatives was reproducibly observed.

Figure 2 shows a typical excitation power dependence of the UV and vis emissions from the TrpMe solution. The intensity of the UV emission with the 340-nm filter (open circles) was dependent on a power law relationship of the excitation intensity, $I_{exc}^{2.79\pm0.03}$ (solid line) in an excitation range from 10 to 60 mW and saturated above 150 mW. This confirms that the UV emission is attributed to the three-photon process. However, the vis emission was observed and changed sensitively with different excitation powers (filled symbols). The emission intensity varied according to about the seventh power of the excitation intensity, $I_{exc}^{6.6\pm0.1}$ (dashed line). The vis emission of the TrpMe solution below about 30 mW varied approximately according to the third power of the excitation intensity. This is considered to be the crosstalk of the fluorescence of TrpMe above 400 nm because the fluorescence intensity is not negligible in the range of the vis detection filter. The vis emission above 150−160 mW was saturated. The exponent of the excitation power dependence changed from 6.3 to 7.1 during experiments. The exponent was dependent on the fitting range because the saturation at the higher excitation range and the contribution of the crosstalk of the three-photon-excited UV emission bring about a smaller value of the exponent. In addition, the uncertainty of the data, the fluctuation of the laser power, and the pulse width resulted in the variation of the exponent. The average of the exponents was 6.8 ± 0.4 with 12 separate measurements when the fitting was performed using data points that yielded the maximum value of the exponent (mostly in the range of 40−120 mW excitation). The possibility of the existence of other minor photoprocesses, which brought about the noninteger value of the exponent, cannot be excluded. However, the emission intensity varied according to the seventh power of the excitation intensity within this experimental error.

Although the slope was independent of the concentration, the emission intensity increased with increasing concentration. The vis emission intensity of 0.1 mM TrpMe excited at 60 mW, about 1.5 kcps, was about 1 order weaker than the emission from the 5HT solution, about 10 kcps, under the same conditions. This confirms a 1 order weaker intensity of the vis emission of tryptophan compared with that of 5HT in the previous report.[7] The power dependence of the 5HT emission (data not presented) was relatively small, $I_{exc}^{5.6\pm0.2}$. This result also confirms the I_{exc}^{5-6} dependence that was reported previously.[7,9] The noninteger value of the exponent might have suggested the small contribution of other processes.

Figure 3 shows the intensity of the vis emission as a function of the concentration of the TrpMe solution at 80-mW excitation. Its intensity was proportional to the concentration up to 1 mM (solid line). Therefore, the emission did not originate from the molecule−molecule interaction of TrpMe molecules or from the solvent background. To identify the species generating the emission, the intensity fluctuation of the vis emission was analyzed by its correlation function. If the species diffuse in the excitation-detection volume, then the fluctuation of the emission intensity arises from their Brownian motion. Therefore, the correlation function of the emission can be interpreted as the fluorescence correlation function.

Figure 4 shows the correlation function of the vis emission with various concentrations of TrpMe. The decay profiles of the correlation function did not change, although the amplitude of the function decreased with an increase in the concentration of the TrpMe solution as shown in Figure 4. The correlation amplitude is determined by the reciprocal of the number of species, which emitted in the vis emission, in the observed volume. Therefore, the concentration of the species increased with an increase in the concentration of TrpMe. The number can be estimated from the fitting to the one-component model of the fluorescence correlation function

$$g_D(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_0}\right)^{-1}\left(1 + \frac{\tau/q^2}{\tau_0}\right)^{-1/2} \quad (1)$$

where τ_0, N, and q are the correlation time, the average number of species, and the structure parameter, respectively.[1] The fitting

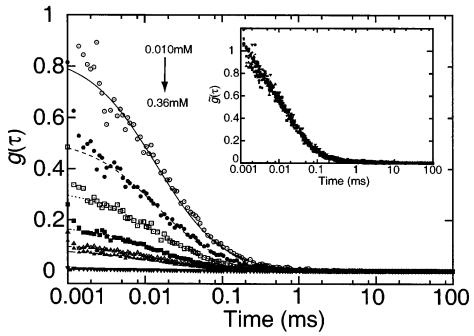

Figure 4. Correlation function of the vis emission intensity with different TrpMe solutions with 80-mW excitation (0.01, 0.02, 0.04, 0.078, 0.15, 0.19, and 0.36 mM in the order indicated by the arrow). The lines are the one-component fitting curves. The inset shows the correlation functions normalized by the amplitude $\tilde{g}(\tau)$.

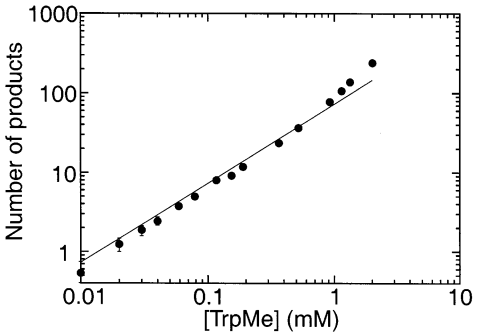

Figure 5. Number of products in the observation volume with different concentrations of TrpMe. The number was obtained by the fitting to the one-component model of the correlation function (eq 1). It is proportional to the concentration of TrpMe (—).

curves are plotted in Figure 4 (lines). The correlation function could not be fit well in a very fast range of the lag time because of the artifact due to the after-pulsing of the photomultiplier tube and because of the statistics of the data. The number of species obtained by the fitting is shown in Figure 5. The number linearly increases with an increase in the concentration, and this result is compatible with the linear dependence of the emission intensity shown in Figure 3. This indicates that the emission is not generated from the medium and that the species emitting in the visible are products, which are freely moving in solution, generated from TrpMe. The concentration of the products can be estimated from the number of products with a volume of observation of 0.75 fL, and the calculation eventually yields about 13 nM in 0.1 mM TrpMe solution at 80-mW excitation. Therefore, it is strongly suggested that about 0.013% of the TrpMe molecules convert a photoproduct that moves freely in the solution.

The correlation functions of the vis emission of 0.1 mM TrpMe with various excitation powers within the range from 60 to 140 mW are shown in Figure 6. The normalized functions are also shown in the inset. The amplitude of the correlation function decreased with an increase in the excitation power and decayed more rapidly. These correlation functions were fit by the one-component correlation function (eq 1), and the number of products was obtained. The number of products and the intensity with different excitation powers are shown in Figure 7. The emission intensity varied approximately according to the seventh power of the excitation power, $I_{exc}^{6.7\pm0.2}$ (solid line). The number of products, however, varied according to about the fifth power, $I_{exc}^{4.9\pm0.1}$ (dashed line). This result suggests that the production of the vis species is mediated by the five-photon excitation process. The brightness of the products, which can

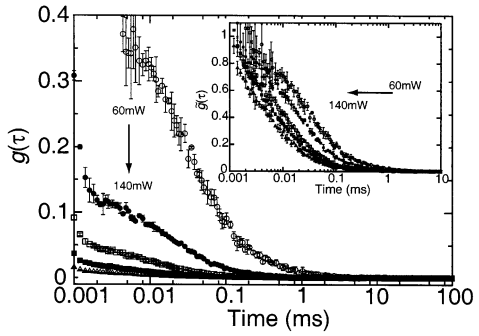

Figure 6. Correlation function of the emission intensity of a 0.1 mM TrpMe solution at various excitation powers (60, 80, 100, 120, and 140 mW in the order indicated by the arrow). The inset shows the correlation functions normalized by the amplitude $\tilde{g}(\tau)$.

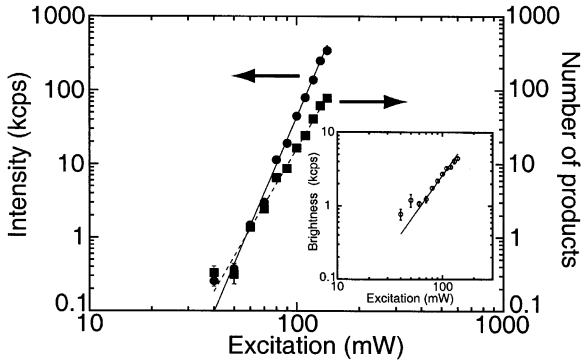

Figure 7. (●) Emission intensity and (■) number of products in the observation volume of a 0.1 mM TrpMe solution. The number of products was obtained by the fitting of eq 1. Best fits with (—) $I_{exc}^{6.7\pm0.2}$ and (- - -) $I_{exc}^{4.9\pm0.1}$. The inset shows brightness of the photoproduct molecule of a 0.1 mM TrpMe solution. (○) The brightness, estimated by the ratio of the intensity to the number of molecules, and (—) the I_{exc}^2 relationship of the excitation power.

be estimated by the ratio of the emission intensity and the number of products, is shown in the inset of Figure 7. The brightness is in good agreement with a square dependence of I_{exc}^2 (solid line). The reason for the deviation from this relationship at low power excitation is the inadequate signal-to-noise ratio of the correlation functions. This result indicates that the vis emission of this species is generated through the two-photon process. Therefore, the vis emission can be considered to be the emission from the two-photon excited state of the product generated from TrpMe through the five-photon excitation process.

The correlation function of the emission rapidly decayed, and the correlation time decreased with increasing excitation power as shown in Figure 6. The correlation time obtained by one-component fitting was 0.026 ms at 60-mW excitation and was significantly decreased to 0.0074 ms at 100 mW. This value, 0.026 ms, is about half of that measured, 0.062 ms, with R123. This 2-fold difference in the diffusion time indicates a factor of 8 difference in the molecular weight if the molecule can be approximated by a spherical shape. This value is unreasonably small because the molecular weight of the R123 cation is only 345. Furthermore, the change in the diffusion time of the products with an increase in the excitation power could not be explained by simple diffusion. Thus, the thermal effect and the photokinetics might contribute the decay of the correlation function.

First, the possibility of the thermal effect should be discussed. The heat generated by the absorption of water is about 5.6×10^{-15} J/pulse with 100-mW 76-MHz excitation using the

extinction coefficient of water of 2.0 m^{-1} at 800 nm.[12] This heat causes an increase of about 0.5 K in a few minutes.[13] Consequently, this increase in temperature due to the absorption of water was negligible in our measurements. The nonlinear absorption of TrpMe also generates heat, and the heat should be proportional to its concentration. If all of the energy of the five-photon absorption, which generates photoproducts of TrpMe, is converted to heat and if all of the TrpMe molecules absorb this energy, then the total energy can be estimated to be about 3.7×10^{-14} J/pulse with a 0.1 mM TrpMe solution. This results in about a 5 K increase in temperature in a few minutes, although this estimation is very rough. Consequently, the heat generation due to nonlinear absorption is not the primary reason behind the smaller value and the change in the diffusion time. This is also supported because the decay of the correlation function with different concentrations from 0.01 to 0.36 mM of TrpMe did not change as shown in Figure 4 (inset). Therefore, it is suggested that the decay is determined not only by diffusive motion but also by photokinetics such as photobleaching and triplet conversion in the volume element.

Because the electronic states of the photoproduct have not been identified, we phenomenologically consider the photokinetics to be the reason behind the change in the correlation decay. The most probable mechanism of the additional fluctuation is the conversion to a nonradiative state from the excited state of the product. This kind of reaction will be classified into two categories of reaction: reversible and irreversible. The fluctuation due to the reversible reaction between the nonradiative state and the radiative excited state through the photocycle contributes an exponential decay of the intensity correlation if the time constant of the photocycle and the lifetime of the nonradiative state are within the same range of diffusion time. The triplet-state conversion, which can be found in many dyes, is a typical example of this type of fluctuation.[14] However, the irreversible reaction that is the conversion to the nonradiative state from the excited state is also regarded as a pseudoequilibrium reaction when the system is large enough. Bleaching is in this category and will also give an additional exponential decay of the correlation function when the conversion time is within a similar range of diffusion time.[15] Nevertheless, the additional exponential decay due to the photokinetics will appear in the correlation function as

$$g(\tau) = g_D(\tau)\{1 - a + a \exp(-\bar{k}_z\tau)\} \qquad (2)$$

where $\bar{k}_z$ is a photokinetic rate constant. $g_D(\tau)$ is the diffusion part of the fluorescence correlation function defined by eq 1. The diffusion part was assumed to be unchanged with various excitation powers and fixed to that with 40-mW excitation. In the fitting, then, parameters $\bar{k}_z$ and a were treated as free parameters. The kinetic rate constants $\bar{k}_z$ are plotted in Figure 8. This constant shows an I^2_{exc} relationship (solid line). More than two exponential decays did not improve the fitting. This fact did not exclude the existence of more than two processes because of insufficient statistics and the after-pulsing distortion of PMT in the fast region of correlation data, which obscured the fast processes. Therefore, there exists at least one process of the conversion to the nonradiative state of microsecond order. This process involves the excited state generated by the two-photon absorption of the products because of the I^2_{exc} relationship of the kinetic rate.

In comparison to the vis emission of 5HT, the emission was assigned to the photoproduct generated by the five-photon process. The intensity of the emission of the 5HT solution, about 10 kcps, was about 1 order more intense than that of the TrpMe solution, about 1.5 kcps, at 60-mW excitation with 0.1 mM solutions in Tris·HCl. The correlation analysis of 5HT and TrpMe yielded that the number of 5HT products, about 11, was 1 order larger than that of TrpMe, about 1.7, under the same conditions. Therefore, the difference in the intensity is attributed to the production rate of visible species. Moreover, this result also indicates that the brightness of the product of TrpMe, about 0.9 kcps, is the same value as that of 5HT, about 0.9 kcps, at 60-mW excitation. The brightness was proportional to the square of the intensity of excitation in both samples. Thus, this result and the similarity of the spectrum strongly suggest that the visible species of TrpMe must resemble those of 5HT.

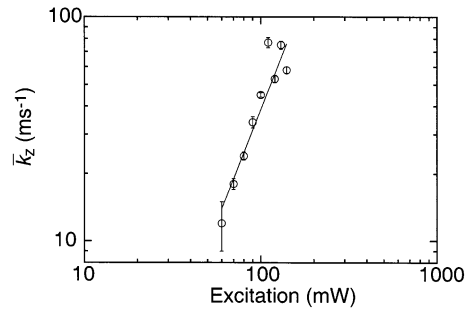

Figure 8. Photokinetic constant vs excitation power. The diffusion time was fixed at 0.036 ms (40-mW excitation), and the kinetic constant was a free parameter in the fitting. (—)Best fit of the I^2_{exc} relationship.

The photophysics and photochemistry of tryptophan and its derivatives are reviewed elsewhere.[16] Many possible paths for relaxation and photochemistry can be considered. Under aerobic conditions, irradiating tryptophan with UV light affords an important photooxidation product, *N*-formylkinurenine, and many possible photoreaction paths for the generation of the products have been proposed. Among the possible reaction paths, the reaction between some active agents, such as hydrated electrons and radicals, and ground or excited states of tryptophan is not the case here because the amount of product was simply proportional to the concentration of TrpMe below about 1 mM, as shown in Figures 3 and 5. At higher concentrations, however, this possibility of a molecule−molecule interaction cannot be excluded because the data points deviated upward and because a new shoulder in the emission spectrum appeared.

The energy of the simultaneous absorption of five photons at 795 nm, 7.78 eV, is larger than the energy separation between S_0 and the excited states, but it might not be possible for this to occur. The triplet−triplet absorption band from the T_1 level is, in the near-UV region from 300 to 450 nm, peaked at about 400 nm.[17] This region is two-photon excitable, and the combination of three-photon excitation to S_1 coincides with five-photon energy. The triplet states and the excited state of the triplet might be involved in the photochemical path of the photoproduct of TrpMe through five-photon excitation. Our preliminary results with additions of potassium iodide showed a remarkable increase in the emission intensity—by about a factor of 4.3—at 60-mW excitation in the presence of 10 mM potassium iodide. Moreover, the amount of product also increased; it was about 8.2 times larger. This result indicates that the production of the visible species is increased with potassium iodide as well as the brightness of the product being quenched. The production path of visible species must involve the triplet state, which is regulated by the balance of the intersystem crossing rate. Detailed comparisons between 5HT and tryptophan should be investigated in detail to understand this photochemistry.

Recently, the fluorescence correlation analysis of tryptophan in hemocyanin, containing 148 tryptophans, was studied with two-photon excitation.[6] This study is the first report of using the fluorescence correlation measurement of the intrinsic fluorescent amino acid residues in proteins. Unfortunately, many proteins contain only a few tryptophan residues, and a further step in the methodology will be needed for the SMD of other proteins because of a 1 to 2 order weaker signal. Our data show that the UV emission of tryptophan with three-photon excitation is very weak, about 10 kcps with a 0.1 mM TrpMe solution at the saturated level as shown in Figure 2. The reason for the weak UV emission of tryptophan is considered to be the loss of the fluorescence yield because of the conversion of TrpMe to the photoproduct through the nonlinear absorption of excitation light. Therefore, the UV emission associated with the three-photon absorption of tryptophan does not meet the SMD condition because of this unfavorable reaction path.

The vis emission is 1 order more intense than the UV emission. The concentration of the vis product estimated from the correlation method was 13 nM ($N = 5.7$) in a 0.1 mM TrpMe solution with 80-mW excitation. This result indicates that the vis emission generated from the "product" with two-photon excitation could be detected at the SMD level. The production rate of the species of the vis emission, however, limits the detection of TrpMe. Therefore, it is still difficult to measure "tryptophan" at the SMD level (∼nM) through this vis emission. Because the characteristics of the vis emission of tryptophan are similar to those of 5HT, the emission intensity will depend on the environment of the molecule. In addition, our preliminary result above suggests that the triplet state of tryptophan is involved in the production. Consequently, the detection limit of tryptophan may be lowered by more than 1 order of concentration with different solvents or additives.

The measurement of the vis emission of the product is at the SMD level, so the emission carries information at the SMD level of tryptophans, which are converted into products, about its environment. For instance, the proteins, which contain the photoproducts generated from tryptophan residues, are fluorescent and detectable at the SMD level. Therefore, the analysis of the vis emission is expected to be applied to a similar analysis of fluorescence correlation of the labeled protein. Eventually, it must give kinetics information about the conformation as well as the interactions of ligands and substrates. The next step is the detection of the vis emission of tryptophan residues in proteins.

Fluorescence correlation spectroscopy is applicable to a wide range of research, including the photochemistry analysis of dyes.[14,18] In comparison with the previous report, this method could apply to the analysis of the mechanism of unknown emissions, such as the visible emission demonstrated here, through the number (i.e., concentration) and the brightness. This is quite a useful approach that can be employed to reveal the new fluorescent product and the mechanism and that allows us to emphasize the importance of the method in photochemistry.

Conclusions

We analyzed the visible emission from a tryptophan methyl-ester solution, which varied approximately according to the seventh power of the excitation intensity. The correlation analysis of the emission indicated that the emission was the two-photon excitation emission of the photoproduct generated through the five-photon process. The generation of the product was facilitated by potassium iodide. The additional fast decay in the correlation function could be attributed to the conversion to the nonradiative state from the excited state of the photoproduct. The novel emission will extend the measurement of tryptophan in proteins and will be applied to its correlation analysis.

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