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Picosecond Fluorescence Dynamics of Tryptophan and 5-Fluorotryptophan in Monellin: Slow Coupled Water-Protein Relaxation Unmasked

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Trp vs. 5FTrp
Abstract

Time Dependent Fluorescence Stokes (emission wavelength) Shifts (TDFSS) from tryptophan (Trp) following sub-picosecond excitation are increasingly used to investigate protein dynamics, most recently enabling active research interest into water dynamics near the surface of proteins. Unlike many fluorescence probes, both the efficiency and the wavelength of Trp fluorescence in proteins are highly sensitive to microenvironment, and Stokes shifts can be dominated by the well-known heterogeneous nature of protein structure, leading to what we call pseudo-TDFSS: shifts that arise from differential decay rates of sub-populations. Here we emphasize a novel, general method that obviates pseudo-TDFSS by replacing Trp by 5-fluorotryptophan (5Ftrp), a fluorescent analog with higher ionization potential and greatly suppressed electron-transfer quenching. 5FTrp slows and suppresses pseudo-TDFSS, thereby providing a clearer view of genuine relaxation caused by solvent and protein response. This procedure is applied to the sweet-tasting protein monellin which has uniquely been the subject of ultrafast studies in two different laboratories (Peon, J. et al, Proc. Natl. Acad. Sci. U. S. A., 2002, 99, 10964; Xu, J. et al, J Am Chem Soc 2006, 128, 1214) that led to disparate interpretations of a 20-ps transient. They differed because of the pseudo-TDFSS present. The current study exploiting special properties of 5FTrp strongly supports the conclusion that both lifetime heterogeneity-based TDFSS and environment relaxation-based TDFSS are present in monellin and 5FTrp-monellin. The original experiments on monellin were most likely dominated by pseudo-TDFSS, whereas in the present investigation of 5FTrp-monellin, the TDFSS is dominated by relaxation and any residual pseudo-TDFSS is overwhelmed and/or slowed to irrelevance.
Introduction

Proteins do the work of life in a largely aqueous milieu, which explains the persistent appearance of studies aimed at protein-water interactions in connection with structure and function of proteins. The sensitivity of fluorescence from the amino acid tryptophan (Trp) is exploited in a high percentage of papers involved in characterizing structure and function of proteins.\(^1,2\) This sensitivity is manifest in several dimensions of fluorescence measurement, but two are most used: the spectral maximum (\(\lambda_{\text{max}}\)) and the transient excited state population, typically characterized by multiple exponential time constants, which also determine the fluorescence quantum yield. Both behaviors depend on time following a short excitation pulse in a way that is unique for each Trp in a particular protein environment.

The availability of stable sub-picosecond excitation pulses, especially in the last decade, has opened rigorous investigation of the 0.5 ps ~ 100 ps time window following excitation, a region that was difficult to access previously.\(^3\) This pioneering work ultimately led to the recognition of a ubiquitous new phenomenon in which the Trp fluorescence spectrum shifts to longer wavelengths with time constants on the 1-100 ps time scale,\(^4,11\) a phenomenon perhaps first glimpsed on the subnanosecond scale by Toptygin et al.\(^12,13\) The time dependent fluorescence Stokes shift (TDFSS) on this time scale is considered "slow" because for free Trp and Trp analogues where the indole ring is nearly 100 % solvent exposed, the corresponding relaxation is typically complete in 2 ps.\(^5,14\) A lively discussion persists regarding the physical basis of the slow TDFSS, mostly regarding whether the slow relaxation reflects primarily the special dynamics of water at the protein surface ("biological water"), or whether protein motions are also being probed.\(^5,11,15-30\)

The sweet-tasting protein monellin was the subject of one of the first studies to invoke slow TDFSS.\(^5\) This protein also has the distinction of being one of the few proteins for which comparable ultrafast studies have been performed by two independent laboratories.\(^5,27\) In the reports from the two studies, quite different interpretations emerged, although the published TRES (Time Resolved Emission Spectra) were quite similar. The earlier report attributed all of the slow TDFSS to the dielectric response of protein surface-associated water, "biological water", to the Trp excited state dipole. The data from the later study,\(^27\) however, was subjected to a global analysis/decay associated spectral (DAS) analysis that evinced no “negative DAS” signature expected for a pure relaxation after 5 ps. Spectral shift was therefore attributed to a phenomenon that in this paper we term “pseudo-TDFSS”, a time dependent shift that is due to population decay heterogeneity;\(^31\) not solvent/protein relaxation. This phenomenon is seldom recognized in the ultrafast fluorescence literature, despite being identified during discussions in the early years by pioneers of TDFSS,\(^32-34\) where it was described variously with terms such as "kinetic hole burning", inhomogeneous lifetime effects, or entirely static, non-relaxation wavelength shift. This effect is ubiquitous, appearing in a high fraction of all studies reporting non-exponential fluorescence decay in glass-like media such as proteins and viscous solvents. In this paper, the term pseudo-TDFSS is not to be confused with intramolecular dual fluorescence involving distant covalently coupled chromophores, which appears in the literature under the keywords "pseudo fluorescence Stokes shift".\(^35-37\) Instead, it is a side result of a phenomenon previously dubbed “Quasi Static Self Quenching” (QSSQ)\(^38,39\): subpopulation(s) of Trp with lifetimes much shorter
than conventional TCSPC instrument limits (e.g., <100ps). QSSQ had been used to account for “missing quantum yield” or aberrant (high) calculated natural lifetimes in nanosecond resolution data. Pseudo-TDFSS is a consequence of those quickly decaying populations contributing photons with energies that differ from the average population. Such a quickly decaying population disappears early, leading to a shift in mean energy without environmental relaxation. Hence, the form of pseudo-TDFSS examined here arises when two (or more) protein subpopulations (discrete species) have different population decay rates and different $\lambda_{\text{max}}$, such that $\lambda_{\text{max}}$ shifts in time only because of the faster disappearance of one of the subpopulations. Pure pseudo-TDFSS has nothing to do with relaxation of the surroundings. This is always a possible mechanism for TDFSS of Trp in proteins when there is heterogeneity in the population decay. Population decay heterogeneity is almost ubiquitous for Trp in proteins, and leads to nanosecond decay profiles that can only be fit with multiple exponential or other models yielding non-exponential decay. For reasons to be discussed later in this paper, pseudo-TDFSS for Trp almost always causes a red shift in time, thereby greatly complicating interpretation if both pseudo-TDFSS and TDFSS are reasonable possibilities. In at least two cases, however, pseudo-TDFSS blue shifts in time, which is an unambiguous indication of pseudo-TDFSS.

The discrimination of heterogeneous vs. relaxation models for ns Trp decay surfaces was extensively debated in the 1980s. One strategy used then was to develop over-determination and spectral association strategies for analysis, leading to the requirement for a “negative DAS signature” (or in phase terms, $m/\cos \phi > 1$) to prove relaxation dominates heterogeneity. This was supplemented with mutagenesis studies in a few cases, seeking to link spectra and kinetics to individual Trp loci. Mutagenesis of Trp surroundings was also done to suppress quenching. Trp analogs were introduced mainly to obtain a native-like probe with desirable (redder) excitation vs. Trp, and reviewed by Ross et al.

Among the emerging Trp analogs, 5-fluorotryptophan (5FTrp) was discovered to incorporate into proteins at nearly 100% efficiency and to have the remarkable property of considerably reducing the appearance of non-exponential decay for Trps that were exhibiting very non-exponential decay. The underlying origin of this behavior has been shown to arise from greatly suppressed quenching by electron transfer due to the inherently higher ionization potential of 5FTrp relative to Trp. This carries the consequences that the quantum yield is higher, and the fluorescence lifetime ($\tau_f$) is much less sensitive to conformational and environmental heterogeneity. There are now many published instances in which such 5FTrp substitution resulted in more homogenous decay than from the native protein Trps. Fortuitously, the fluorescence wavelength sensitivity to solvent polarity for 5FTrp is very nearly the same as that of Trp. This means that suppression of pseudo-TDFSS allows one to interpret remnant TDFSS as relaxation dynamics. In other words, 5FTrp reports almost exclusively relaxation-based TDFSS without interference from ET derived pseudo-TDFSS.

The primary aim of this paper is to exploit these properties of 5FTrp to test for the presence of both pseudo-TDFSS and TDFSS due to water and/or protein relaxation following sub-ps excitation of the single-Trp protein monellin. This was done by comparing the TDFSS of monellin with a mutant monellin in which the Trp has been replaced with 5FTrp.
Figure 1 provides a cartoon to aid visualization of how lowering both the HOMO and LUMO of the indole ring by 5-F substitution can lead to these useful properties. The work reported in this paper exploits this behavior and rationalizes why two laboratories could extract quite different conclusions about monellin dynamics.

**Materials and Methods**

**Production of 5FTrp-monellin.** E. coli Trp auxotroph CY15077 was from the Genetic Stock Center at Yale University. The λDE3 prophage was introduced in CY15077 using a λDE3 lysogenation kit (Novagen), yielding strain CY15077 (DE3). This strain was transformed with vector pET22b+ harboring the single chain monellin MNE1 gene. An overnight culture of this transformed strain in LB medium + 100 μg/ml ampiciline, grown at 37°C and 200 r.p.m. was used to inoculate (1: 40) 1L M9 medium + 100 μg/ml ampiciline and 1 mM Trp. Growth was continued at 37 °C and at OD$^{600} = 0.5$, 0.25 mM IPTG was added. After 10 min cells were pelleted at 3100×g during 10 min. Cells were resuspended in 300 ml M9 medium without Trp, pelleted and resuspended in M9 medium + 100 μg/ml ampiciline, 0.25 mM IPTG and 1 mM DL 5-FTrp (Apollo Scientific). Induction was continued for 5H at 37°C.
For the Trp containing MNE1 protein, the overnight culture described above was used to inoculate (1: 40) 1 L LB medium + 100 μg/ml ampicilime and the culture was incubated at 37 °C and 200 r.p.m. At OD$^{600} = 0.8$, 0.25 mM IPTG was added and culturing was continued for 5H.

**Purification of 5FTrp-monellin.** Both the Trp- and 5-FTrp-labeled MNE1 were isolated as follows: The cell pellets were resuspended and washed in 25 mM sodium acetate buffer pH 5.5 and disrupted with a French Press as described (2). After centrifugation, the supernatant was partly purified by Resource S (GE Healthcare) chromatography with 25 mM sodium acetate pH 5.5 as Buffer A, and this buffer containing 1.0 M NaCl as Buffer B. Monellin elutes at about 100 mM NaCl and the protein was concentrated by Centricon MWCO = 3 kD concentrators (Millipore). Further purification was achieved by gel filtration chromatography using a Superdex75 column (GE Healthcare) with 10 mM sodium phosphate pH 7.2. At this low salt concentration monellin has non-specific interaction to the column material and elutes as a 3.0 kDa protein ($V_e = 16.0$ ml).

When an elution buffer of 25 sodium acetate pH 5.5 with 150 mM NaCl was used monellin elutes at 13.9 ml, corresponding with a 9.5 kDa protein. Trp- and 5-FTrp-labeled monellin were concentrated by centrifugal filter devices (Centricon MWCO = 3 kD, Millipore) to 0.7 (≈ 65 μM) and 5.3 mg/ml (≈ 490 μM), respectively. The 5-FTrp incorporation efficiency (92%, see Figure S1) was determined using mass spectroscopy as described.

The final concentrations of proteins were determined by absorption measurement using the extinction coefficient $\varepsilon_{277} = 1.46 \times 10^4$ M$^{-1}$ cm$^{-1}$. A fresh sample solution was prepared for each time-resolved measurement.

**Sub-picosecond fluorescence measurement.** A mode-locked Ti: sapphire laser (Tsunami, Newport) pumped by a diode-pumped solid state laser (Millennia Pro, Newport) was used to generate a $\sim 350$ mW pulse train with a typical pulse duration of 120 fs at a repetition rate of 82 MHz and the central wavelength of 885 nm. These pulses were selected with a Pockels cell for seeding a Ti: sapphire regenerative amplifier (Spitfire, Newport). The amplified pulses were switched out of the cavity with pulse energy of $\sim 140$ μJ and an autocorrelation pulse width of 350 fs at a repetition rate of 5 kHz. 295 nm ultraviolet excitation with an average power up to 30 mW was obtained from a sequential 1 mm BBO crystal and 0.5 mm BBO crystal for doubling and tripling, respectively. Two dichroic mirrors were used to separate the UV beam (tripled) from the infrared beam (fundamental), and blue beam (doubled). Before excitation of the sample, the power of our UV beam was carefully attenuated to $\sim 1$ mW to avoid undesirable photochemistry, hole burning, and other undesirable effects. The protein solution was held in a circular array of thin cells (T-20, NSG Precision Cells) with a path length of 1 mm in a continuously (>5 m/s) spinning delrin stacked slotted disk. The residual fundamental pulse at 885 nm was retro-reflected from a hollow cube corner on a computer-controlled precision linear stage, and this variably delayed pulse was used as a gate pulse for the upconversion process. The fluorescence emission was collected via off-axis parabola and focused into a 0.2 mm thick BBO mixing crystal, and the upconversion signal was produced via type I sum frequency generation with the gate pulse in the crystal. To reject the strong background signals (infrared laser, remnant UV and unconverted fluorescence) accompanying the upconverted beam, a non-collinear configuration was arranged between infrared laser and fluorescence. Polarizations of gated fluorescence were determined by the orientation of nonlinear crystals; no added linear polarizer was used. The polarization of the excitation beam was chosen by a servo-controlled zero-order half-wave plate. Hence there were no elements that induce polarization bias (for anisotropy calculation, Grating factor~1.). By angle tuning the mixing crystal, the upconverted fluorescence, with a wavelength in the range 230-280 nm, always
polarized, was directed into a monochromator (Triax 320, Jobin Yvon Inc. bandwidth of 0.5 nm) and a solar blind photomultiplier tube (R2078, Hamamatsu, dark count < 1 Hz. Amplified SBPMT signals were discriminated and recorded by a gated single photon counter (994, EG&G Ortec). Photon arrival events were held to less than 5 percent of the repetition rate to minimize “pileup”. The “lamp” (AKA “apparatus” or “instrument response”) function was determined by measuring the cross-correlation traces (routinely, and more precisely) between UV-generated ordinary Raman scattering in water and the infrared pulse. In both ways, the lamp function was found to be around 450 fs (FWHM), with a timing jitter of less than 30 fs. When collecting on longer time scales, detuning to ~ 1 ps FWHM was occasionally done to optimize other parameters. Instrument polarization bias was verified with the linear fluorophore p-terphenyl, which yielded an initial anisotropy of 0.40+/-.01 and a single rotational correlation time of 41 ps in neat cyclohexane (both from Sigma/Aldrich).

100 picosecond - 20 nanosecond fluorescence measurement. A tunable Spectra-Physics Model 3520 cavity dumped dye laser was synchronously pumped by a frequency-doubled, mode-locked NdYVO4 laser (Vanguard 2000-HM532, Newport). The dye laser output was doubled into the ultraviolet via a modified Inrad autotracker with BBO crystal. For the present work, rhodamine 6G / glycerol was the laser dye used at 590 nm, and magic angle emission/vertical excitation was employed at 295 nm with pulses having a FWHM < 2 ps. The fluorescence was recorded from 310 nm to 405 nm via JobinYvonH10 monochromator with 8 nm bandwidth and a cooled Hamamatsu MCP photomultiplier. The aggregate instrumental response time width was about 100 ps, so measurement of lifetimes of 50 ps and greater is possible in this instrument. Melatonin in water was used as a monoexponential standard. Lifetimes were obtained by fitting the decay data to a multiexponential model, according to the photon-weighted NLLS nonlinear least-squares method. Goodness of fit was deduced from inspection of residuals and their autocorrelation and \( \chi^2 \) functions. (the fits employed in this work yielded values of 1.01-1.3). For decay-associated spectra (DAS), a time-resolved decay surface was obtained on Monellin by excitation at 295 nm and observation every 5 nm over the emission band. The instrument response function was contemporaneously obtained with a light-scattering suspension of dilute colloidal silica. Alternating the sample with the ’lamp’, stepping of the monochromator, data collection, and transfer of data from the Ortec MCB analyzer to the computer was done automatically (program “DAS32”). In the analysis of the multiple curves obtained for a DAS, all were satisfactorily fit to the same global triple-exponential model.

Steady-state absorption and fluorescence spectra were recorded with a diode array spectrophotometer (HP 8452A) and Fluorolog-3 spectrophotofluorometer (Horiba-SPEX), respectively. The DAS and TRES were calibrated by normalizing our steady state spectrum of Trp in water to spectra taken on another fluorometer previously referenced to a standard blackbody.

QM-MM Simulations. Starting with PDB code 1MOL, including crystallographic waters, the protein was solvated with ~11,000 TIP3P explicit water molecules, with the requirement that no protein component was any closer than 12 Å from any box surface. This was satisfied by cubic periodic boundary conditions. Steepest descent minimization was applied to each of the structures, followed by equilibration at 300 K using GROMACS4.5.4[62] and the OPLS force field for a total of 1.2 ns, with a dynamics time step of 2 fs. Short-range van der Waals and electrostatic cutoffs
were set at 10 Å. Particle Mesh Ewald was used for long-range electrostatics. Details for constructing the starting pdb geometry for 5FTrp monellin are provided in Supporting Information.

For both structures, a 30-ns ground state simulation using default ground state topology files was performed, from which coordinates were extracted every 100 ps. The latter were then used as starting points for 300 1-ns direct-response TDFSS curves, simulated by instantaneously switching the charges of the Trp3 residue from their default ground state values to those of the \( ^1\text{L}_a \) state. Following excitation, the system was evolved in response to the changed chromophore charges for 1 ns, during which coordinate snapshots were saved at 0.1 ps intervals.

As described previously,\(^{30,63,64}\) each MD frame of coordinates was used to perform Zerner’s INDO/S-CIS\(^{65}\) spectroscopically parameterized quantum mechanical calculations (“Zindo”) on a chromophore that contains the Trp side chain and both adjacent backbone amides. The remainder of the protein and water atom point charges served as the sole perturbation by the environment.

![Figure 2](image.png)

**Figure 2** Bars showing relative fluorescence quantum yields from 3-methylindole (3MI) in three different environments for which quenching by electron transfer is believed to be the important factor in determining the quantum yield. Top (pink) is for indole ring, and bottom (blue) is for 5-fluoroindole ring. This provides a graphic comparison of the effect of nearby amides on the quantum yield of Trp and 5FTrp.

Crucial for this study, Zindo fluorine atom parameters appear effective at reproducing the special inductive effect produced by 5-F substitution. The HOMO is lowered by 0.23 eV, similar to what would be Koopman’s theorem prediction for the experimentally observed ionization potential relative to that of Trp, while at the same time predicting a slight red shift for the fluorescence.
Results

Figure 2 provides quantitative information regarding the effect of 5FTrp substitution on the quantum yield ($\Phi_f$) of 3-methlyindole (3MI), which may be considered the basic chromophore for Trp in proteins), N-acetyltryptophanamide (NATA, a widely used model for Trp in proteins), and Trp in the protein monellin due to quenching by nearby amides and side chains. For NATA it is seen that $\Phi_f$ is reduced by 60% relative to 3MI because of electron transfer to the two amides, but for 5F-NATA, $\Phi_f$ is reduced by only 16%. For monellin, quenching is somewhat greater, with $\Phi_f$ being reduced by 77% relative to 3MI, but only by 18% for 5FTrp-monellin. This strongly suggests that pseudo-TDFSS, if present, will be greatly attenuated in 5FTrp-monellin relative to monellin.

Note also that 5-fluoro substitution apparently increases the intrinsic non-radiative decay rate of the 3MI ring, under the reasonable assumption that the radiative rate is not changed significantly, with the consequence that the measured non-quenched fluorescence lifetime of 5-F3MI is 6.8 ns in water compared to 9.1 ns for 3MI.

Next we compare the fluorescence spectra of monellin and 5FTrp-monellin at different times following excitation, as reconstructed from TDFSS decay curves measured at single wavelengths. Figure 3A indicates a ~15% decrease in excited state population in the interval 10-30 ps; this is consistent with a population decay of 16 ps. During the same interval, the corresponding decrease in excited state population for 5FTrp-monellin is seen in Figure 3B to be only.

![Figure 3](image-url)  
**Figure 3** Comparison of time gated fluorescence spectra for monellin (A) and 5FTrp-monellin (B). Figure 3A indicates a ~15% decrease in excited state population in the interval 10-30 ps; this is consistent with a population decay of 16 ps. During the same interval, the corresponding decrease in excited state population for 5FTrp-monellin is seen in Figure 3B to be only.

Next we compare the fluorescence spectra of monellin and 5FTrp monellin at different times following excitation, as reconstructed from TDFSS decay curves measured at single wavelengths. Figure 3A indicates a ~15% decrease in excited state population in the interval 10-30 ps; this is consistent with a population decay time of 16 ps. During the same interval, the corresponding decrease in excited state population for 5FTrp-monellin is seen in Figure 3B to be much less, provided one examines the area under the curves. For 5FTrp-monellin the increase for wavelengths > 356 nm is nearly the same as the decrease for wavelengths < 356 nm. This implies
that a fast electron transfer quenching that was present for the WT monellin is largely absent for 5FTrp-monellin.

Figure 4 compares decay associated spectra (DAS) for WT and 5FTrp monellin. DAS is a common format for displaying lifetime data, which commonly assigns the non-exponential decay seen at each wavelength to a small number of discrete conformers that do not interconvert during the excited state lifetime. The fluorescence from individual conformers (or families of conformers sharing the same lifetime) decay mono-exponentially. A best fit is obtained by globally fitting with the constraint that the set of decay constants is independent of wavelength; I(\(\lambda, t\)) = \(\sum \alpha_i(\lambda) e^{-t/\tau_i}\). The pre-exponential weighting factors (alphas) depend on wavelength, and define the DAS. DAS are, by definition, the spectra associated with decay times. They are not always associated with conformers; in fact, the characteristics of DAS in systems undergoing relaxation or other “excited state reactions” are well known. DAS are crucial for identifying pseudo-TDFSS vs. genuine TDFSS. We also considered another approach for detecting heterogeneity, “TRANES”, but our DAS data are truncated (especially on the red side), making it difficult to reconstruct TRES with sufficient precision to normalize. We consequently hope to improve the range of our next upconversion instrument, but that is beyond the scope of this manuscript.

Employing 5FTrp, a dramatic difference is seen: the ~16 ps DAS (Figure 4A) is always positive for normal monellin whereas from 5FTrp-monellin is strongly negative on the red side of the spectrum (Figure 4B). A negative DAS component (alpha) is caused by the rising intensity found in the decay curve found at wavelengths > ~360 nm, as shown in Figure 5B. When the fluorescence spectrum of a single species red-shifts as a function of time with negligible population decay, the intensity decreases swiftly at short wavelengths and increases correspondingly at long wavelengths. To create a rising rather than falling exponential means the alpha will be negative.

Figure 4 Comparison of DAS for monellin (A) and 5Ftrp monellin (B).
at long wavelengths. In the case of pure pseudo-TDFSS, i.e. when there is heterogeneity but no relaxation-based TDFSS, no DAS would exhibit negative alpha at any wavelength.

The crucial signatures leading to negative DAS regions comes from the decay profiles as a function of wavelength, which are shown in Figure 5. In contrast to the absence of a rising signal after 5ps found by Xu et al. for WT enzyme, shown in Figure 5A, the 5Ftrp monellin decay profiles show a strongly rising signal in time at wavelengths > 360 nm. These “rise time” components were not observed for WT monellin at even longer wavelengths.

**Figure 5** Ultrafast decay curves observed at different wavelengths for WT (A) at 400 nm and 5Ftrp monellin (B) at 333(purple), 340 (black), 350(red), 363 (blue) and 375(green) nm. 5Ftrp monellin has a 16ps rising exponential term in its transient fluorescence when emission wavelength is > 360nm. These “rise time” components were not observed for WT monellin at even longer wavelengths.

The crucial signatures leading to negative DAS regions comes from the decay profiles as a function of wavelength, which are shown in Figure 5. In contrast to the absence of a rising signal after 5ps found by Xu et al. for WT enzyme, shown in Figure 5A, the 5Ftrp-monellin decay profiles show a strongly rising signal in time at wavelengths > 360 nm. These can be caused by a "slow" relaxation process due to nearby water and charged or polar protein elements, or their coupled

**Figure 6** Comparison of decay associated spectra for WT (A) and 5Ftrp monellin (B) on the ns time scale, constructed from TCSPC. Both have three exponential decay terms, but they differ drastically in the component amplitudes while showing similar lifetimes. Note that the short-lived decay (0.7ns) in 5Ftrp has very low amplitude at wavelengths >350nm.
motion. The positive alpha for ~16-ps electron transfer-based pseudo-TDFSS quenching found for normal Trp in WT monellin likely dominated and “masked” this slower relaxation process.

We look now at DAS for WT and 5FTrp-monellin on the ns time scale, constructed from time correlated single photon counting (TCSPC). These are displayed in Figures 6 A and B respectively. Both have three exponential decay terms, but these differ drastically in the component amplitudes while showing similar lifetimes. In effect, there is a reversal of the dominance of 0.7 ns and ~5 ns components. For WT, the long lived (5.2 ns) component is almost negligible but it becomes the major component in the 5F mutant. The 0.7 ns component contributes nearly 40% for WT, but < 10% for 5FTrp-monellin, and it has very low amplitude at wavelengths >350nm (suggesting a relaxation origin). These results are in line with the quantum yields for these two proteins. The similarity of contribution from the 2.5 ns component is interesting, and will be addressed in the Discussion.

![Figure 7](image-url) Comparison of simulated direct response of TDFSS from the average of 300 1 ns MD + QM trajectories for WT and 5Ftrp monellin in Tip3p explicit water. The 300-ps component becomes evident when the full 2ns trajectory is shown. (See Figure S2).

To help interpret the experimental results, we have performed classical molecular dynamics (MD) simulations followed by semi-empirical quantum mechanical (QM) computations on monellin and 5FTrp-monellin solvated by 11,000 explicit waters. Following lengthy equilibration and production in which Trp atomic charges were the default ground state values, excitation to the $^1L_a$ excited state (the fluorescing state) was simulated by switching the charges to the average values given by the QM, and continuing the simulation for 1 ns while storing coordinates every 0.1 ps. Figures 7 A and B show the average over 300 trajectories of the Zindo computed Ground-->$^1L_a$ transition energy as a function of time following “excitation” for WT and 5FTrp monellin. Despite the large number of independent trajectories, the remaining large standard deviation is testimony to the extreme fluctuations present in the time dependent transition energy. As found in an earlier investigation, in addition to ~1 ps component, a ~20 ps decay component is required to fit the computed TDFSS in both cases. A comparably sized component of ~300 ps is also required for fitting the relaxation simulation out to 2 ns (see Figure S2, Supporting Information).
No attempt is made in this work to compute the pseudo-TDFSS directly. Indirect computational evidence that 5FTrp substitution will reduce lifetime heterogeneity and raise the quantum yield, however, comes from the computed CT and $^1L_a$ transitions energies, for which the CT-$^1L_a$ gap is increased by an average of 2500 cm$^{-1}$. See Figure S3, (Supporting Information).

**Discussion**

The results presented above are entirely consistent with the established pattern found for replacement of Trp by 5Ftrp in a number of proteins, peptides, and Trp itself. Namely, the average quantum yield is higher, and the heterogeneity of lifetimes is less pronounced, with the longest lifetime being close to that of the unquenched case. This means that the degree in which the observed TDFSS is contaminated by pseudo-TDFSS will be much diminished, thereby allowing the relaxation-based TDFSS to dominate. Taken together, therefore, the monellin and 5FTrp-monellin results show that the pseudo-TDFSS and relaxation-based TDFSS have nearly the same time constant of ~16-20 ps. The accuracy of upconversion TDFSS experiments is such that if one effect is dominant, credible detection of the minor component is effectively not possible.

5FTrp substitution is, therefore, a complementary approach to the mutation of proteins to remove nearby electron-abstracting sinks (“quenchers”)\(^7\), as it changes the protein structure only at the Trp sidechain instead of the “nearest neighbor” Trp environment. The evaluation of which perturbation is more conservative with regard to protein dynamics will require extensive simulation of each particular protein, but both can be successful approaches to separate pseudo-from genuine TDFSS.

**Possible Structural concerns with 5FTrp monellin.** There is no structural information for 5FTrp-monellin, prompting concern that perturbation of replacing the H with F at position 5 could cause local structural changes, which conceivably might dominate effects we are attributing to electron transfer only. Comparing the MD simulations for the two forms, we find that the detailed structure near Trp3, shows that 5FTrp occupies the same pocket with very similar water exposure as with the WT protein. At a more detailed level, however, the orientation of the dipole of the N-terminus ammonium-Glu2 COO- is generally reversed for 5FTrp monellin, putting the COO- near the Trp amide, which one would naively think would also favor a higher quantum yield. This protein contribution is apparently compensated by opposite contributions from water, as evidenced by our computational finding that replacement of F with H at position 5, while keeping the fields and potentials the same as dictated by the 5FTrp-monellin simulations, lowers the lowest CT state by an average of 1300 cm$^{-1}$—consistent with Zindo F parameters, which appear effective at reproducing the inductive effect. (The HOMO is lowered by 0.23 eV, similar to experiment.)

**Blue shifting pseudo-TDFSS.** To emphasize the ubiquitous nature of pseudo-TDFSS for solvent sensitive probes in glassy environments, we review two examples for which this phenomenon could not be confused with environmental relaxation. Unlike relaxation-based TDFSS, pseudo-TDFSS can give rise to shifts of $\lambda_{\text{max}}$ to either longer (red-shift) or shorter (blue-shift) wavelengths, depending on whether the lower energy discrete species decay more slowly or more rapidly, respectively. In fact, the ground-breaking investigations by Frauenfelder and coworkers on geminate recombination of photo-dissociated CO-myoglobin,\(^43\) wherein the now well-accepted heterogeneity of kinetic processes in proteins was firmly established, pseudo-TDFSS was seen as
crucial to understanding of a time-dependent blue shift of the near IR CT transition of myoglobin. This was termed “kinetic hole burning” by Agmon.\textsuperscript{33,34} Also at that time, Agmon made the reasonable suggestion that the pioneering observations of Fleming, Maroncelli, and coworkers on the TDFSS of coumarin 153, attributed entirely to solvent relaxation, might also contain a substantial pseudo-TDFSS component. Maroncelli et al. replied with an eloquent, broadly informative comment that removed any doubt that what was observed at room temperature was indeed solvent relaxation alone.\textsuperscript{32} These authors, however, also acknowledged that they had observed pseudo-TDFSS in glassy solvents at lower temperatures.

More recently, another well documented and well explained account of blue shifting (and therefore unequivocally pseudo-TDFSS) was observed for the artificial fluorescent amino acid aladan, mutagenically placed in various environments of the protein GB1 by the Boxer group.\textsuperscript{44} Aladan apparently has an environment-sensitive fluorescence quantum yield and $\lambda_{\text{max}}$, contrasting with that of Trp by having a longer lifetime in non-polar environments than in polar environments. The TDFSS for aladan in the majority of sites in GB1 was found to red shift during the first half of decay, and then dramatically blue shift during the last half. This was explained by relaxation-based TDFSS from the more quickly decaying aladan in more polar environments dominating the early time fluorescence, with the surviving population of aladan in more non-polar sites dominating the latter part of the decay.

**Summary and Conclusions**

The motivation for the work presented here was three-fold: (1) to bring attention to the concept of pseudo-TDFSS, a ubiquitous phenomenon in ultrafast time-resolved measurements, known for over 20 years in the chemical literature, yet relatively unfamiliar; (2) to present substitution of 5-fluorotryptophan as a general method for distinguishing pseudo-TDFSS from the more familiar relaxation-based TDFSS; and (3) to illustrate how the method reconciles the disparate interpretations of TDFSS for the protein monellin.

At the nanosecond level, 5FTrp substitution in monellin increases the average fluorescence lifetime and quantum yield of protein fluorescence by increasing the contribution of long lifetime components (4-5 ns) at the expense of the short lifetime components (0.7 ns), while the intermediate component (2.5 ns) is relatively unchanged. The extent of lifetime heterogeneity is therefore considerably reduced.

At the picosecond level, comparing the time dependence of both population and $\lambda_{\text{max}}$ of monellin in which the single Trp has been replaced with 5-fluorotryptophan (5FTrp) uncovers TDFSS that was obscured by pseudo-TDFSS in the case of WT monellin. Given the properties of 5FTrp, we now believe that both lifetime heterogeneity-based TDFSS and environment relaxation-based TDFSS are present in monellin. The original experiments on monellin\textsuperscript{5} were most likely dominated by the former, noted pseudo-TDFSS here. In the present investigation of 5FTrp-monellin, the TDFSS is dominated by environment relaxation, and any slow pseudo-TDFSS is largely obscured. MD + QM computations of TDFSS from this study are consistent with these findings.\textit{Separation of these effects is valuable because both pseudo- and genuine TDFSS are useful probes of protein dynamics; the former gives us insight into the very near neighbor interaction between Trp and electron acceptors, the latter between the Trp excited dipole and the local milieu of coupled water...}
and protein dipoles. Some water is loosely coupled “bulk” water yielding dynamics near 2ps, and some are apparently moving in tens of picoseconds. The slow coupled process shows a robust balance between protein and water dipoles.\(^{30}\) 5FTrp (and likely other analogs) thus adds another “over-determination” scheme to help us dissect protein-water dynamics.

**Associated Content**

**Supporting Information**
Figures S1–S4 and details of MD for 5FTrp monellin. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**
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**References**

Supplementary Information for:

Picosecond Fluorescence Dynamics of Tryptophan and 5-Fluorotryptophan
in Monellin: Slow Water-Protein Relaxation Unmasked

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Overview

Here we present Figures S1-S4, details for constructing a 5FTrp monellin pdb file for use
in QM-MM computations, results of measuring the time dependence fluorescence anisotropy of
the two proteins studied, and a discussion of possible proton-transfer quenching of the 5FTrp
fluorescence in the 5FTrp monellin.
Figure S1. Maldi-TOF spectrum of the tryptic peptide GEWEILDIGFTQLGK of monellin NME1 containing Trp (theoretical mass = 1917.15 Dalton) or 5-FTrp (theoretical mass 1935, 14).
Figure S2. Comparison of simulated direct response of TDFSS from the average of 300 1 ns MD + QM trajectories for WT monellin in TIP3p explicit water, showing the 300-ps component.
Figure S3. Averages of 300 trajectories each INDO/S-CIS computed Ground→\(^1\)La and Ground→ CT transition energies (720,000 calculations), and the CT-1La gap computed from these from MD simulations of monellin and 5FTrpmonellin. The consistently higher gap of \(~2500\) cm\(^{-1}\) is consistent with shutting down most electron transfer from 5FTrp compared to Trp. The larger gap comes from a combination of higher CT energy and lower 1La energy for 5Ftrp.
Details for constructing a 5FTrp monellin pdb file for use in QM-MM computations.

Appropriate entries in the forcefield files for bond length (CZ3-FZ3), angles (CH2-CZ3-FZ3 and FZ3-CZ3-CE3) and the dihedral were generated. A new residue for 5-F-tryptophan species (TRF) was also generated. The pdb file was then generated by replacing TRP with TRF in the 1MOL.pdb structure and following the normal procedure. The angles were set at 120 degrees and the dihedral was set at 180 deg. The C-F bond length was set at 1.36 Å based on Gaussian HF/6-311G and HF/3-12G calculations, which gave 1.381 Å and 1.364 Å respectively, comparing with that reported for monofluorobenzene (1.354 Å) by S. Doraiswamy and S.D. Sharma, (1983), J. Mol. Struct. 102, 81-92.

Time Dependence of Fluorescence Anisotropy for Monellin and 5Ftrp monellin

To further document the authenticity and integrity of the 5Ftrp monellin construct, we have measured the time dependence of the fluorescence anisotropy on the nanosecond time scale, shown in Figure 8. The much lower initial anisotropy for 5Ftrp monellin is a signature of all 5-position substitutions of indole. It is caused by a pronounced raising of the HOMO -1 energy relative to other MO energies due to strong overlap of the F and C₅ π atomic orbitals arising from the high electron density at the 5 position for this MO combined with the large amplitude of excitation from the HOMO-1 MO in the ¹Lₐ state.¹,² The net effect is to reduce the separation of the ¹Lₐ and ¹L₉ states, while keeping ¹Lₐ the fluorescing state. As a result, excitation near 295 nm initially excites a higher fraction of ¹L₉ state compared the unsubstituted ring,¹ creating a low initial anisotropy (“r₀”) because of the orthogonal nature of the ¹Lₐ and ¹L₉ transition moment directions.² This is
consistent with steady state anisotropy values found when exciting 5FNATA vs. NATA at 295 nm in glassy solvent.\textsuperscript{3}

Unfortunately, initial anisotropy approaching 0.10 serves to deprive disc-like fluorophores from the ability to sense “in plane” rotation.\textsuperscript{4} Extensive anisotropy decay studies at multiple excitation wavelengths will thus be needed to fully compare Trp and 5FTrp motions.

\textbf{Figure S4} Comparison of nanosecond fluorescence anisotropy (r) vs. time for WT and 5Ftrp monellin. The lower initial anisotropy value is consistent with steady state anisotropy values when exciting at 295 nm. Version with lamp function and fit available.
Proton transfer quenching.

The 2.6 ns DAS component appears to be unchanged by 5-F substitution. This certainly could be simply coincidental; on the other hand, one might speculate that this component reflects only proton transfer quenching, for which 5-F substitution apparently has no effect.\(^5\) Such quenching plays a major role in the free Trp amino acid, but has rarely been documented in protein environments. Indeed, there is at least one example of the absence of proton transfer quenching in the protein environment when Trp is in contact with two lysines.\(^6\) In monellin, our MD simulations show that Lys44 spends much time 3.7-4.2 Å from CZ2 (ring position 7), which is a hot spot for proton exchange.\(^7,8\) Despite definite local conformational differences induced by the 5-F group according to our MD results, the ammonium group of Lys44 again hovers over CZ2 of the 5FTrp in the fluorinated monellin.

This is consistent with two reported instances in disordered or unfolded\(^9,10\) 5FTrp proteins in which average lifetimes ranged from 2.5 to 3.4 ns, i.e., shorter than the unquenched value near 5 ns. In both cases, there is an abundance of –NH\(^3+\) groups from lysines nearby. Further

Table 1. Fluorescence Anisotropy parameters

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<th>(\beta_1)</th>
<th>(\beta_2)</th>
<th>(\phi_1)</th>
<th>(\phi_2)</th>
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<td>WT Monellin</td>
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<td>(\backslash)</td>
<td>1.54</td>
<td>(\backslash)</td>
<td>1.00</td>
</tr>
</tbody>
</table>
experiments that alter the proton donating and accepting environment of this Trp or measure nmr proxies for excited state protonation directly\textsuperscript{7,11} will be needed to test this conjecture.

Reference List


