Ultrafast Polarized Fluorescence Measurements on Tryptophan and a Tryptophan-Containing Peptide

Olaf F. A. Larsen,*,† Ivo H. M. van Stokkum,† Anjali Pandit,‡ Rienk van Grondelle,† and Herbert van Amerongen†,§

Division of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands, Department of Structural Biology, Faculty of Earth and Lifesciences, Vrije Universiteit Amsterdam, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands, and Laboratory of Biophysics, Department of Agrotechnology and Food Sciences, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

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In this work polarized picosecond fluorescence measurements were performed on isolated tryptophan and tryptophan in a small 22-mer peptide using a streak camera coupled to a spectrograph as a detection system. In both cases the fluorescence decay was multiexponential with decay times of ∼500 ps and ∼4 ns. Surprisingly, also a short-lived (∼16 ps) isotropic fluorescence component was found for both tryptophan and the peptide which, to our knowledge, has never been observed before. Fluorescence anisotropy data showed rotational correlation times of 155 ps and 1.5 ns for the peptide, reflecting local and overall peptide dynamics, respectively. Recently it was argued [Lakowicz, J. R. *Photochem. Photobiol.* 2000, *72*, 421] that the nonexponential fluorescence decay of tryptophan in proteins is mainly due to spectral relaxation, whereas for isolated tryptophan it is due to different rotamers. Our results do not support this view. In contrast we conclude in both cases that the different fluorescence decay times should be ascribed to different rotameric states.

Introduction

For many years it has been recognized that both the structure and dynamics of biomolecules are directly linked to their functioning.[1] To study these two properties, fluorescence spectroscopy has been widely applied. For proteins, use can be made of the three native fluorescent amino acids phenylalanine, tyrosine, and tryptophan. Of these three amino acids, tryptophan (Trp) is the most popular one because its chromophoric group (indole) possesses the highest extinction coefficient as well as the highest fluorescence quantum yield.[2] Furthermore, one of the two lowest-energy singlet states (the ^1L_a state) is very sensitive to changes in its direct environment (for a review see ref 3), providing the possibility to distinguish between hydrophobic and hydrophilic environments. However, the interpretation of Trp fluorescence data is often obscured because of the complicated photophysics. The lowest energy absorption band at 280 nm is due to two electronic transitions; the excited singlet states ^1L_a and ^1L_b. The fluorescence mainly originates from the ^1L_a state, but ^1L_b fluorescence has also been observed.[4] Because of the small energy spacing between these excited states, rapid interconversion between them can take place, resulting in a fast (nonrotational) contribution to the fluorescence anisotropy decay.[5,6]

In addition to this interconversion between the ^1L_a and the ^1L_b states, the fluorescence of Trp is also complicated by the existence of several rotameric states arising from either rotation around the protein backbone $C_\alpha-C_\beta$ axis, giving rise to 3 different "χ_1" conformers, and rotation of the indole group around the $C_\beta-C_\gamma$ axis, resulting in 2 so-called "χ_2" rotamers.[7] Contrary to the monoexponential fluorescence decay observed in isolated indole, a multiexponential fluorescence decay is observed for Trp with each decay time assumed to originate from an individual rotameric state. The reason for the multiexponential decay of the fluorescence is supposedly that the positively charged amino group interacts with the indole ring, giving rise to quenching because of an electron-transfer process.[11] Molecular dynamics simulations performed by Engh et al.[7] resulted in the expectation of finding two rotameric χ_2 states on the fluorescent ns time scale, while the three other χ_1 rotamers were supposed to be equilibrating very rapidly on the time scale of fluorescence. However, Gordon et al.[8] pointed out that rapid equilibration of either χ_1 or χ_2 rotamers depends on the method of how the hydrogen atoms are treated in the MD simulation. The MD studies give better agreement with experimental data if the modeling is based on slow interconversion of the χ_1 rotamers and rapid interconversion of the χ_2 rotamers.[9] Another argument that favors "slow" χ_1 rotamers is that rapid equilibration of the χ_1 rotamers would result in the loss of the chirality of the C_α atom, resulting in both χ_2 conformers experiencing the same environment in Trp. This would lead to a monoexponential fluorescence decay, which is not observed experimentally.[8] Time-resolved polarized fluorescence studies by Dahms et al.[10] on a crystal of a small peptide gave additional evidence for the multiexponential fluorescence decay to be originating from different rotamers of the Trp residue.

The multiexponential fluorescence decay of (single) Trp proteins has for a long time been ascribed to the presence of different rotamers as well. However, in a recent review by Lakowicz, an alternative opinion has been given. In this review,

* Corresponding author. E-mail: larsen@amolf.nl. Telephone: +31-20-6081264. Fax: +31-20-6684106. Current address: FOM-Institute for Atomic and Molecular Physics (AMOLF), Kruislaan 407, 1098 SJ Amsterdam, The Netherlands.

† Division of Physics and Astronomy, Vrije Universiteit Amsterdam.

‡ Department of Structural Biology, Vrije Universiteit Amsterdam.

§ Department of Agrotechnology and Food Sciences.

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it is claimed that spectral relaxation is the main cause for the observed multiexponentiality of the fluorescence.[11] The main argument is that the amino and carboxyl groups of Trp that reside in a protein are uncharged (because they are involved in the formation of the peptide backbone). Thus, instead of the fluorescence of Trp, the fluorescence of *N*-acetyl-L-tryptophanamide (NATA) is actually monitored. NATA is Trp in which the amino group is acetylated and the carboxyl group is amidated. For NATA, a single fluorescence lifetime is expected and also observed.[11] Observation of multiexponential fluorescence decay of Trp in proteins should, according to Lakowicz, then mainly be explained by spectral relaxation. Spectral relaxation is suggested by the fact that the mean fluorescence decay times increase with increasing emission wavelength and that collisional quenching often results in a shift of the emission to shorter wavelengths. Another observation that favors spectral relaxation is that the emission maximum shifts to longer wavelengths upon shifting the excitation to longer wavelengths.[12] The emission can be quenched by the peptide backbone,[13] by water,[14] and by excited-state electron and excited-state proton transfer with certain amino acids.[15]

In this work, the polarized fluorescence of Trp and Trp in a small peptide has been studied. The peptide that has been investigated consists of the RNA binding site of the HIV-1 REV (regulator of the expression of virion proteins) peptide. This REV peptide is known to bind to the so-called RRE (REV responsive element), an RNA sequence originating from HIV-1. The structure of the REV−RRE complex has been resolved in detail using NMR spectroscopy.[16] The REV peptide of HIV-1 plays a crucial role in the regulation of protein synthesis for the virus.[17] The sequence of the 22-mer peptide we used in this study is:

aaaTRQARRNRRRRWRERQRaa

The alanines on both ends are not present in the wild-type peptide but were attached to induce more α-helicity, which is known to be important for specific binding to the RRE.[18] Use has been made of a streak camera combined with a spectrograph as a detection system. In contrast to more commonly used techniques such as single-photon counting and fluorescence upconversion, the decay of the complete emission spectrum can be monitored over a large time interval, ranging from a few picoseconds up to a few nanoseconds. Using global analysis,[19] we are able to obtain both short-lived (picosecond) as well as long-lived (nanosecond) fluorescence components with their corresponding decay associated emission spectra.

Experimental Section

Tryptophan (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in a buffer (pH = 7.3) consisting of 10 mM K_2HPO_4/KH_2PO_4 pH = 7.5 buffer, with 100 mM potassium fluoride (KF). The optical density was 0.2/cm at 300 nm. The peptide was dissolved in the same buffer as described for Trp, with an optical density of 0.25 at 300 nm.

Excitation was performed by the frequency-doubled output of an optical parametric amplifier (Coherent), which was pumped by an amplified titanium−sapphire laser (Coherent). The polarization of the excitation pulses (centered at 300 nm, pulse width ~100 fs, repetition rate 40 kHz or 125 kHz) could be adjusted either parallel or perpendicular to the detection polarization using a Berek polarization compensator. Using a Glan-Thompson polarizer, the vertically polarized emission was detected. The emission light was led into a spectrograph (Chromex) and monitored using a streak-camera system (Hamamatsu) coupled to a CCD camera (Hamamatsu).[20,21] This system enabled us to measure the decay of the complete (polarized) emission spectrum with a time response of ~4 ps FWHM. The fluorescence decay of the whole emission spectrum could be measured over a time regime of 2.2 ns (full time window of the streak camera). With each polarization condition, three different time bases were used (200, 800, and 2200 ps). The instrument response depended on the time base used and ranged from ~4 to ~25 ps. By making use of the "back-sweep" of the synchroscan system, decay times longer than 2.2 ns could also be estimated.[20] The spectral resolution was ~8 nm.

Excitation was performed at the very edge of the cuvette, to prevent inner-filter effects that might arise because of the high optical density of the sample. All experiments were performed using a temperature-controlled cuvette holder thermostated at 21 °C.

Data Analysis

The data have been analyzed by a global analysis method.[19] The two fluorescence components I_{VV} and I_{HV} (vertically and horizontally polarized excitation, both with vertically polarized detection) were fitted to

$$\begin{bmatrix} I_{VV}(t) \\ I_{HV}(t) \end{bmatrix} = \begin{bmatrix} I_{iso}(t)(1 + 2r(t)) \\ I_{iso}(t)(1 - r(t)) \end{bmatrix} \otimes I(t) \quad (1)$$

where $I_{iso}(t)$ is the isotropic fluorescence signal, and $r(t)$ is the fluorescence anisotropy. $I(t)$ reflects the instrument response and ⊗ denotes convolution. A shift parameter, which was dependent upon wavelength and direction of polarization, was included in the instrument response in order to model dispersion effects.

The isotropic decay, $I_{iso}(t)$, is described by a sum of decays, each component having its own decay associated spectrum (DAS) $A_i(\lambda)$ and fluorescence decay rate k_i:

$$I_{iso}(t) = \sum_{i=1}^{n} A_i(\lambda)\, e^{-k_i t} \quad (2)$$

To describe the Raman scatter in the REV22 data, an instantaneous component $I(t)$ was added. This component contributes most in the parallel data, and its associated spectrum peaks at ~330 nm. Isotropic decay times were obtained from a simultaneous fit of all three timebases (200, 800, and 2200 ps) together.

The anisotropy $r(t)$ is described with a nonassociative model as the sum of individual anisotropy components j, each with its own rotational correlation time τ_j and amplitude r_j:

$$r(t) = \sum_{j=1}^{m} r_j\, e^{-t/\tau_j} \quad (3)$$

All obtained isotropic decay times and rotational correlation times, with corresponding amplitudes, have a precision better than 10%.

We also performed an analysis of the data in which each isotropic fluorescence contribution was linked to an associated anisotropy time. However, the signal-to-noise ratio did not allow to obtain meaningful conclusions.

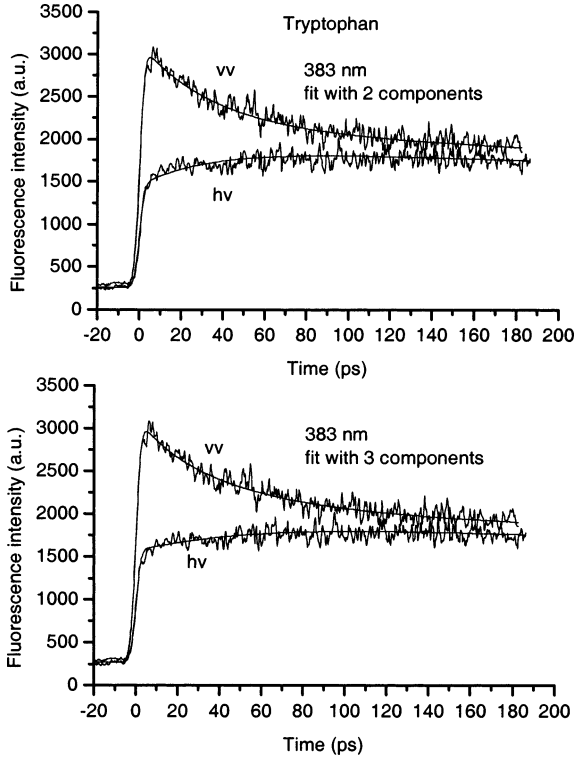

Figure 1. Representative fluorescence decay traces of Trp at 383 nm fitted with, respectively, two and three isotropic decay components (fit: smooth lines); vv and hv are vertically and horizontally polarized excitation, respectively (eq 1).

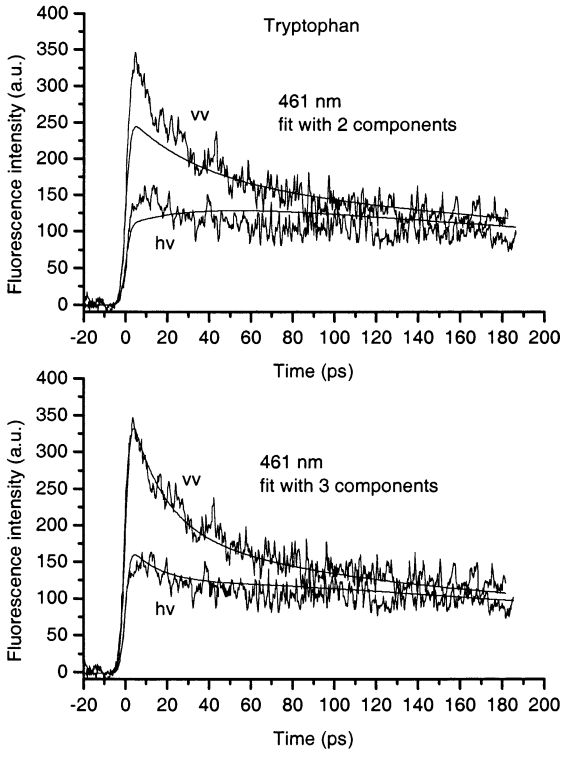

Figure 2. Representative fluorescence decay traces of Trp at 461 nm fitted with, respectively, two and three isotropic decay components (fit: smooth lines); vv and hv are vertically and horizontally polarized excitation, respectively (eq 1).

The center of gravity of the emission (λ_{cg}) for each DAS was calculated following eq 4:

$$\lambda_{cg} = \frac{\sum_{\lambda=315}^{500} \lambda\, I(\lambda)}{\sum_{\lambda=315}^{500} I(\lambda)} \tag{4}$$

with λ the wavelength (in nm) and $I(\lambda)$ the corresponding emission intensity (a.u.) at λ. Analogous to this, the center of gravity of the overall emission spectrum at time t for both Trp and REV22 was calculated from the DAS according to

$$\lambda_{cg}(t) = \frac{\sum_{i}\sum_{\lambda=315}^{500} A_i(\lambda)\, e^{-k_i t}\lambda}{\sum_{i}\sum_{\lambda=315}^{500} A_i(\lambda)\, e^{-k_i t}} \tag{5}$$

where $A_i(\lambda)$ are the DAS and k_i the corresponding decay rates.

Results

The fluorescence-decay kinetics of Trp was fitted using three isotropic lifetimes (~13 ps, 500 ps, ~3.8 ns) and one anisotropy time of 44 ps. Such a fast isotropic decay component of ~13 ps has to our knowledge never been observed before. The corresponding DAS has a higher amplitude in the longer wavelength part of the spectrum when compared to the two other "slow" components. To illustrate the presence of this fast red component, several representative traces are given. Figures1 and 2 show fluorescence decay traces of Trp at 383 and 461 nm with the global fitting results using, respectively, two or three isotropic decay components. Clearly, the data cannot be fitted satisfactorily without the use of the extra (third) fast component at the red side of the spectrum (e.g., 461 nm), while this is not necessary for shorter wavelengths (e.g., 383 nm). It should be noted that the traces as shown below are obtained from a fit of the 200 ps time base only, because this time base possess the fastest time response (~4 ps). Because the "slow" decay times cannot be accurately estimated from this time base only, they are fixed. Hence, a fit using two fixed (slow) components is compared with a fit using two fixed (slow) components and a "free" fast component (the slow components were already obtained from a simultaneous fit of all three time bases together).

The fluorescence decay of REV22 could also be described using three similar isotropic lifetimes of, respectively, ~19 ps, 541 ps, and ~3.5 ns. Again, a fast "red" component is observed (~19 ps). To illustrate the presence of this fast red component for REV22 as well, similar traces as for Trp are given in Figures 3 and 4.

The decay associated emission spectra for Trp and REV22 obtained from the global analysis with their corresponding centers of gravity are shown in Figure 5. The spectra are very similar for Trp and REV22.

The centers of gravity (λ_{cg}) of the overall emission as a function of time were calculated for both Trp and REV22 according to eq 5. The result is shown in Figure 6.

The Trp data were fitted with a rotational correlation time of 44 ps (amplitude 0.26). The analysis of the data for REV22 resulted in two rotational correlation times of, respectively, 155 ps (amplitude 0.09) and ~1.5 ns (amplitude 0.20). The fitting results of Trp and REV22 are summarized in Table 1.

Discussion

Recently, time-resolved fluorescence polarization studies have been performed on small peptides which were composed of

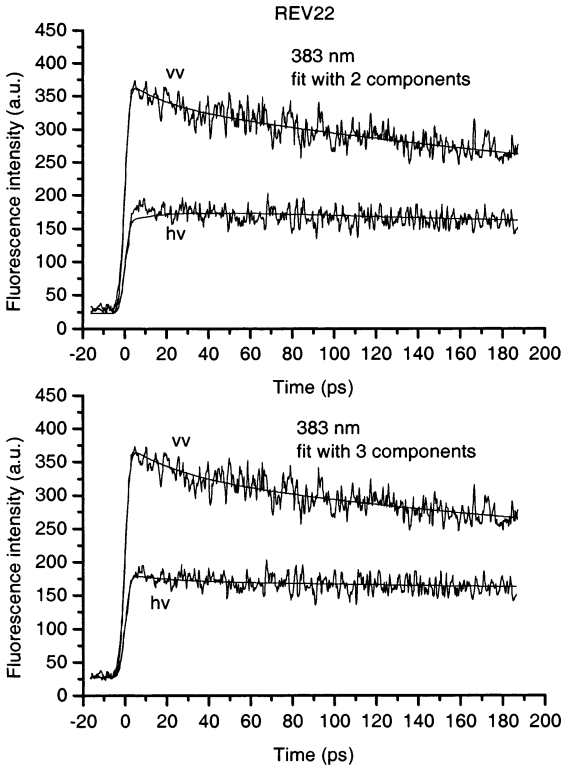

Figure 3. Representative fluorescence decay traces of REV22 at 383 nm fitted with, respectively, two and three isotropic decay components (fit: smooth lines). vv and hv: vertically and horizontally polarized excitation, respectively (eq 1).

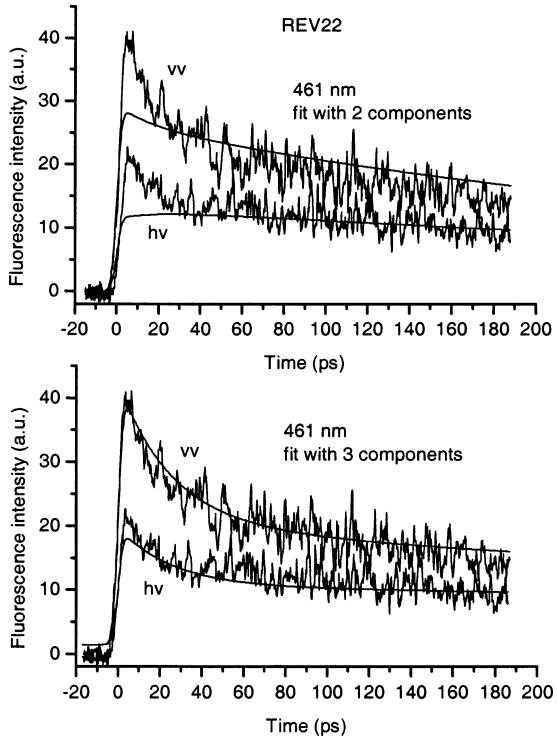

Figure 4. Representative fluorescence decay traces of REV22 at 461 nm fitted with, respectively, two and three isotropic decay components (fit: smooth lines); vv and hv are vertically and horizontally polarized excitation, respectively (eq 1).

small parts of the RNA binding domain of the REV peptide.[25] For the peptide that was composed of the same sequence as in our experiments, but with an extra glutamine on the C-terminus and without the extra attached alanines that we used, similar isotropic data were obtained except for the 19 ps component.

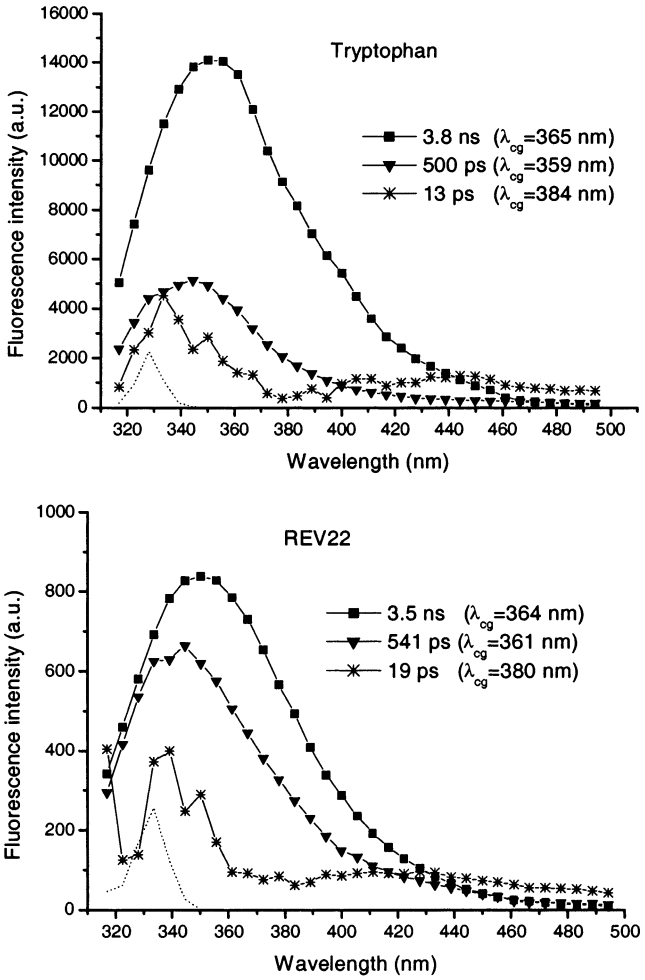

Figure 5. Decay-associated emission spectra of, respectively, Trp (top) and REV22 (bottom); λ_{cg} is the center of gravity (eq 4). The dotted spectra indicate the position of the Raman peak.

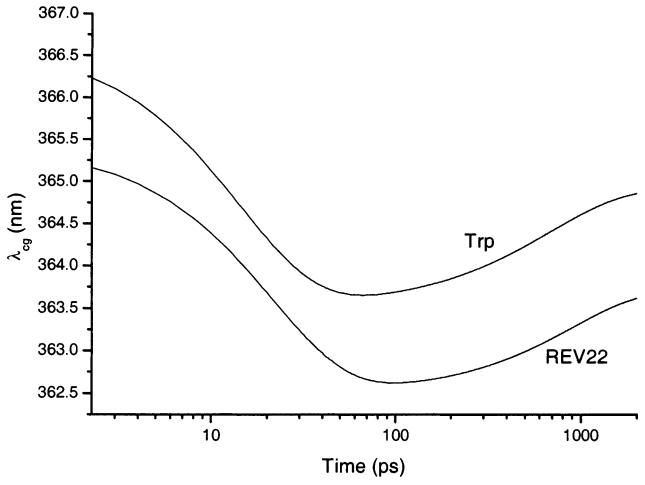

Figure 6. Center of gravity of the emission (λ_{cg}) of Trp and REV22 as a function of time.

The short "red" isotropic component in our experiments is not found in the data by Lam et al.,[25] probably because the time resolution of their single photon counting setup is not sufficient. The buffer that was used in those experiments had similar pH (7.5) but a different salt composition.[25] Three isotropic components were extracted using global analysis. Similar to our experiments, a component of 443 ps was found (541 ps in our experiments), with a similar contribution to the signal of 20% (34% in our experiments). Two long lifetime components of

TABLE 1: Lifetimes (in ps) Resulting from Global Analysis of Trp and REV22[a]

molecule	$1/k_1$ (%)	$1/k_2$ (%)	$1/k_3$ (%)	τ_1 (r_1)	τ_2 (r_2)
Trp	13 ± 3 (16)	500 (20)	3794 (64)	44 (0.26)	
REV22	19 ± 3 (18)	541 (34)	3501 (48)	155 (0.09)	1526 (0.20)

[a] The percentages represent the relative contributions of the components to the total signal (as determined from the areas of the corresponding DAS), while for the rotational correlation times the amplitudes of each component are also given.

2.01 ns and 4.61 ns were found. The three isotropic times were also interpreted as originating from different rotameric states from the tryptophan.[26] In our data, only one ns time was found. However, it is difficult to extract two ns isotropic times from our measurements, considering the restricted time range of the streak camera synchroscan unit (2.2 ns, although the "backsweep" enables us to estimate longer times). The rotational correlation times are also comparable: 143 ps and 1.10 ns with respective contributions of 0.066 and 0.148, versus 155 ps and ~1.5 ns with respective contributions 0.09 and 0.20 for our data. The long "overall" rotational correlation time of the peptide we studied is somewhat higher, indicating a slightly slower overall rotation, which is expected because of the extra alanines in our case. The measurements were obtained at nearly the same temperatures (20 °C and 21 °C, respectively).

The isotropic fluorescence decay we find for Trp is characterized by three time constants: ~13 ps, 500 ps, and ~3.8 ns. The two slower isotropic times are similar to the 0.51 ns and 3.1 ns obtained by Szabo and Reyner.[22] These two lifetimes were attributed to different rotameric states of Trp. Estimates of the spectra for these different conformers were obtained earlier, measuring the fluorescence decay of Trp at different wavelengths (e.g., ref 22). However, with our setup, and the application of global analysis, we were able to obtain these emission spectra "directly". The big advantage of the streak-camera setup is that the decay traces over the entire emission wavelength range can be probed simultaneously, in contrast to other techniques such as fluorescence upconversion and single-photon timing where only one detection wavelength at a time can be probed.

Interestingly, the fast (~13 ps) component has significant amplitude in the long-wavelength part of the emission spectrum. This component has to our knowledge not been observed before. It is unlikely that this time constant is due to $^1L_a - ^1L_b$ equilibration. This equilibration time was already estimated before to be 1.6 ps,[6] which cannot be resolved with the current signal-to-noise ratio. Furthermore, the estimated initial anisotropy is far below the theoretical limit of 0.4,[28] which is the expected value before electronic equilibration and solvation take place. The equilibration between ^1L_a and ^1L_b is known to result in a nonrotational contribution to the anisotropy decay of 2 ps,[6] leading to strong depolarization that cannot be resolved in this experiment. Spectral relaxation is ruled out because this process should result in a red shift of the emission,[11] whereas in this case the opposite is observed. Because the fast fluorescence lifetime cannot be attributed to either $^1L_a - ^1L_b$ excited-state equilibration or spectral relaxation, we propose that also this lifetime originates from a rotamer of Trp.

The emission characteristics of the REV22 peptide (with λ_{cg} at 364, 361, and 380 nm, respectively, for the DAS) are highly reminiscent of those of Trp alone (respectively, 365, 359, and 384 nm). Again, two "slow" isotropic lifetimes are found, namely 541 ps and ~3.5 ns. Although the maxima of these spectra are different, they are reminiscent of the A and B states as proposed by Hansen et al.[23] (emission maxima of, respectively, 335 and 355 nm), who attributed these times to different rotameric states of Trp. The corresponding DAS of REV22 are very similar to those of Trp, though the contributions of the two states to the total signal are (somewhat) different (for Trp these are 20% and 64% for the 500 ps and 3.8 ns times, respectively, and 34% and 48% for the REV22 peptide). Also in the peptide, a third very fast isotropic time (~19 ps), with a corresponding "red" decay associated emission spectrum, is observed. The contribution of this component is very similar to that of Trp in solution (18% in Rev22 and 16% in Trp). The contribution of the fast component for the peptide is stronger in the red part of the spectrum than for Trp in solution. The spectral dynamics induced by this fast decay component is illustrated in Figure 6 for both Trp and REV22. From this plot it can be seen that the behavior of the center of gravity of the emission for both Trp and REV22 expose very similar behavior; first a fast blue shift is observed, followed by a slower red shift. This initial fast blue shift favors the idea that the different fluorescence components originate from different rotamers, and not from spectral relaxation as proposed earlier.[11] If spectral relaxation was responsible for the emission behavior in the peptide, only a red shift would be observed and no initial blue shift.

Another argument favoring the fluorescence components of REV22 originating from different rotamers is the extracted "fast" rotational correlation time of REV22. Two rotational correlation times are obtained from the data: 155 ps (amplitude 0.09) and ~1.5 ns (amplitude 0.20). It is natural to assign the long time to the overall peptide dynamics, whereas the shorter time probably reflects the local Trp dynamics. The ns component is indeed of the same order of magnitude as the value for the rotational correlation time of 900 ps when the peptide is considered as a sphere.[2] The 155 ps anisotropy time is much shorter than for other single Trp proteins. Lakowicz mentions a ~500 ps "fast" anisotropy time for several studied single Trp proteins.[11] In this paper,[11] it is reasoned that the ~500 ps isotropic lifetime is originating from spectral relaxation that is induced by "fast" structural dynamics reflected in the ~500 ps anisotropy time. However, the fast rotational correlation time we find (155 ps) is much faster than the isotropic ~500 ps; no correlation between the isotropic decay times and the anisotropic decay times as suggested by Lakowicz is found. Similar results have been found for ultrafast fluorescence studies on the single-Trp peptide melittin.[29]

Conclusions

The photophysics of Trp and of Trp in a small peptide has been investigated using polarized fluorescence spectrosopy with picosecond time resolution. The time evolution of complete (polarized) emission spectra has been recorded. Both Trp alone and Trp in the peptide exhibit a multiexponential isotropic fluorescence decay. This multiexponentiality has been associated with ground-state heterogeneity due to different rotamers of Trp. The corresponding decay associated spectra of these rotameric states have been extracted from the data. In addition to ~500 ps and ~4 ns lifetimes, a very fast lifetime of ~16 ps was found for both Trp and the peptide. The decay associated emission spectrum of this fast component is shifted to longer wavelengths as compared to the emission spectra of the two other components. The fast disappearance of this red component leads to a fast initial blue shift of the spectral center of gravity and strongly indicates that the different isotropic fluorescence components originate from different rotamers of Trp and not from spectral relaxation for which only a red shift is expected. For the peptide,

two anisotropy times are found of, respectively, 155 ps and 1.5 ns, reflecting local Trp dynamics and overall peptide dynamics. No isotropic fluorescence component with a decay time similar to the fast rotational correlation time could be extracted from the data, which also disfavors a spectral relaxation model.

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