Application Note

Time-Resolved Spectroscopy of Proteins

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Introduction

Optical absorption of proteins in the region of 250-300 nm (near-UV) is governed by the aromatic amino acid residues phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) [1]. Compared with Tyr and Trp the absorbance of Phe in the near-UV as well as its quantum yield is negligible (Fig. 1A). Consequently, the observed protein fluorescence is mainly caused by Trp and Tyr residues. When excited between 290-295 nm the emission of proteins is generally dominated by the Trp fluorescence due to the large difference between the extinction coefficients (Fig. 1B) and the circumstance that Tyr fluorescence is often efficiently quenched by carbonyl groups of the peptide backbone or neighboured residues.

Trp fluorescence is a highly sensitive probe for changes of the protein´s secondary and tertiary structure. Depending on hydrophobicity of the surrounding of a Trp residue the center of its emission band can vary over several tens of nanometers as well as its quantum yield and life-times changes. Therefore, steady state spectroscopy studies even with proteins containing several Trp residues can provide insight into conformational changes upon e.g. unfolding, substrate binding or other catalytical processes. Furthermore, single Trp mutants provide the possibility to examine protein dynamic at individual sites of the protein with time-resolved fluorescence spectroscopy. In this application note we demonstrate the potential of nowadays commercially available lifetime spectrometers and mention the limitations of such a spectrometer with sub-nanosecond resolution for determining dynamic processes in proteins.

Set-up and Data Anylsis

All experiments were performed with the compact fluorescence lifetime spectrometer FluoTime 100 (PicoQuant) equipped with polarizing optics suited for the near-UV region. The samples were excited at 290 nm with the sub-nanosecond pulsed LED PLS 290 (PicoQuant) at a repetition frequency of 10 MHz driven by the PDL 800-B (PicoQuant). Possible parasitic red emission of the PLS 290 was blocked using a suited bandpass filter (FF01-310/25, Semrock Inc.). The system was equipped with a photomultiplier tube and the data acquisition was

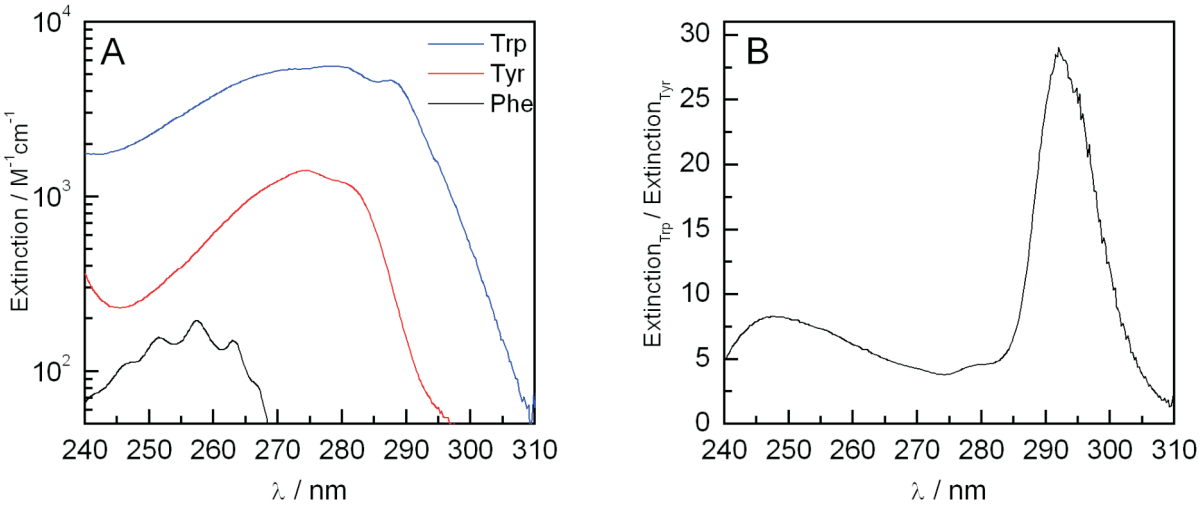

Fig. 1: (A) Extinction of the 3 aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) in the near-UV and (B) the ratio of the extinction of Trp and Tyr (data taken from [2]).

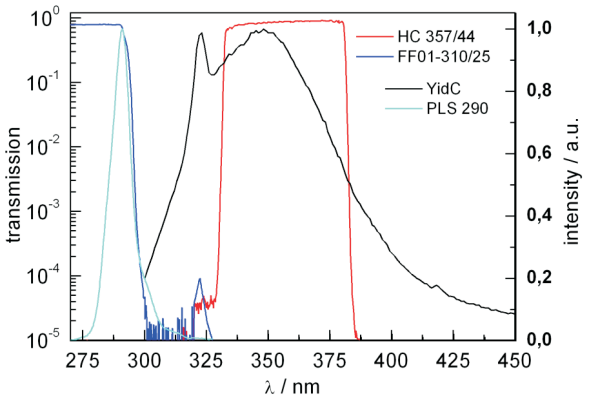

Fig. 2: Fluorescence emission spectra of the single Trp mutant YidC$_{W508}$ (excited at 290nm) and the pulsed LED PLS 290 (both spectra are normalized) together with the transmission profiles of the filters used in the experiment.

done using the PicoHarp 300 TCSPC module (PicoQuant). The instrument response function (IRF) of the complete system was measured with the stray light signal of a dilute colloidal silica suspension (Ludox ®) and had a FWHM of 700 ps, limited by the pulse width of the PLS 290.

Raman scattering of the 290 nm excitation pulse causes an emission line centered at 322 nm which is often not negligible (compared to the intensity of the single Trp residue) and should be filtered out in order to avoid distortions of the measured fluorescence decays. With our protein samples Raman emission is observed on the blue edge of the emission spectrum. By mounting a HC 357/44 bandpass filter (AHF Analysentechnik) in the emission pathway, the Raman line could be effectively blocked (see Fig. 2).

All measurements were analysed with the FluoFit software package (PicoQuant). Fluorescence decays were fitted with the exponential model

$$I(t) = \int_{-\infty}^{t} \mathrm{IRF}(t') \sum_{i=1}^{n} I_i \exp\left(-\frac{t-t'}{\tau_i}\right) dt' \qquad (1)$$

where I_i is the amplitude and τ_i is the lifetime of the *i*-th decay component. Fluorescence anisotropy were calculated with

$$r(t) = \frac{I_{VV}(t) - G\, I_{VH}(t)}{I_{VV}(t) + 2\, G\, I_{VH}(t)} \qquad (2)$$

where I_{VV} and I_{VH} correspond to the intensity decays measured at vertically polarized excitation and vertically and horizontally, respectively, polarized emission. The G-factor accounts for the polarization-dependent sensitivity of the instrument. For our set-up the G-factor was 1,0. The anisotropies *r(t)* were then fitted with the simple exponential model

$$r(t) = \sum_{i=1}^{n} r_i \exp\left(-t/\theta_i\right) \qquad (3)$$

where r_i is the anisotropy and θ_i is the correlation time of the *i*-th component.

By inspection of the residuals and their autocorrelation functions the goodness of all fits were checked. It turned out that at least 2 but never more than 3 free parameters (decay times) were needed to reach a fitting result with sufficient quality ($\chi^2 < 1.2$).

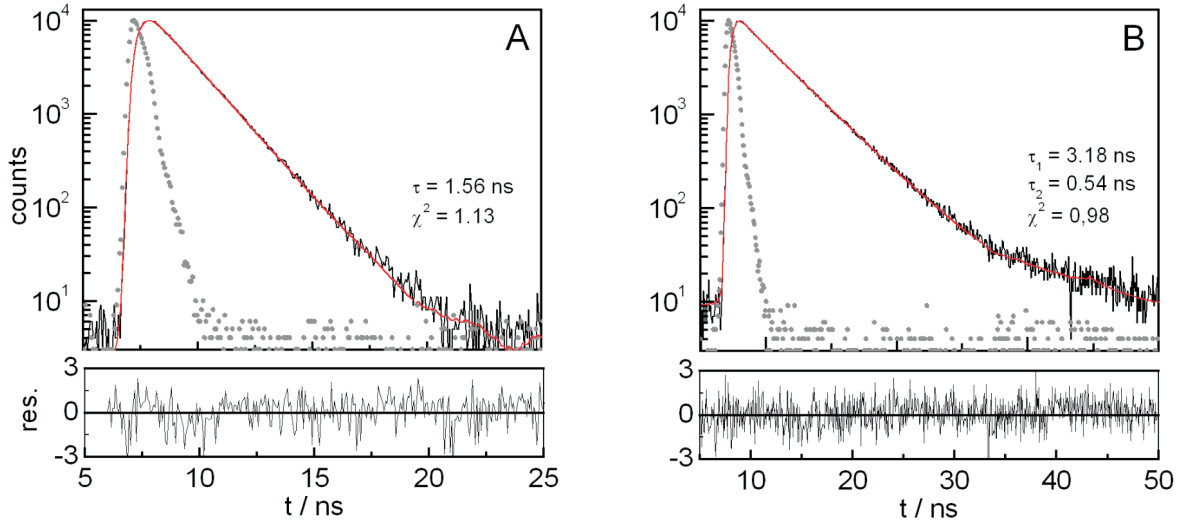

Fig. 3: Fluorescence decay (black curves) of (A) PPO in degassed ethanol and (B) Trp in phosphate buffer at pH 7.0 and the corresponding IRFs (dotted curves). The fitted decays (red curves) and the weighted residuals are for (A) a single-exponential and (B) a double-exponential fit. The best-fit values for the decay times and χ^2–values for each fit are noted. All measurements were done at 20°C with a time resolution of 64 ps.

Tab 1: Fluorescence lifetimes of PPO, tryptophan an related compunds

Sample	Lifetime (ns)		Fractional amplitude 3	
	τ_1	τ_2	α_1	α_2
PPO[1]	1,56	-	1	-
NATA[1]	2,98	-	1	-
Trp[2]	3,18	0,54	0,79	0,21
NATE[1]	1,76	0,68	0,72	0,28

PPO was dissolved in degassed ethanol; Trp, NATA and NATE in 10 mM phosphate buffer at pH 7.0. All measurements were done at 20°C.

[1] without emission polarizer

[2] emission polarizer at "magic-angle"

[3] $\alpha_i = I_i / \Sigma I_i$

Lifetime standards

As near-UV single lifetime standards 2,5-diphenyl-oxazole (PPO) and the Trp analog N-acetyl-L-tryptophan amide (NATA) were used. As an example the fluorescence decay of PPO is shown in Fig. 3A. The calculated lifetimes are presented in Tab. 1.

Tryptophan itself is usually not used as a lifetime standard due to the circumstance that its two lifetimes and their fractional amplitudes strongly depend on several parameters, e.g. the excitation wavelength, the optical filter and the pH-value. However, we also measured the lifetimes of free Trp (see Fig. 3B) and its analog N-acetyltryptophan ethyl ester (NATE). The results for both compounds, also shown in Tab. 1, are within the range of values found in the literature. All lifetime data in Tab. 1 are in good agreement with the literature values [1, 3].

In order to obtain correct intensity decay times it is necessary to measure with the excitation polarizer set to vertical and the emission polarizer set at the "magic-angle" (54,7°) or, as a good approximation, without the emission polarizer. Otherwise the components I_{VV} and I_{VH} are not be properly weighted and as a result the recovered lifetimes would be incorrect.

Protein samples

In this study we investigated the interaction of the E. *coli* bacterial membrane insertase YidC with its substrate Pf3 coat. YidC is a 61 kD, six spanning membrane protein which contains 11 Trp residues at the periplasmic interface [4] whereas Pf3 coat is a 4.6 kD, single-spanning protein with one Trp residue at its C-terminus [5].

In our experiment a single Trp mutant $YidC_{W508}$ was reconstituted into semi-synthetic lipid vesicles and titrated with Pf3W0t coat, a Trp less mutant of Pf3 coat. The proposed membrane topology of YidC is displayed in Fig. 4A where also the position of the single Trp residue is indicated.

It was previously shown that YidC binds reversibly to Pf3 coat with a dissociation constant of about 1 μM as well as a conformational change at the periplasmic interface of the insertase is induced by this interaction [6, 7]. An artistic view of this process is given in Fig 4B. We will present in the next section that such a conformational change manifests itself in a change on the correlation times of the Trp residue at position 508.

Experimental Data

In the last 3 decades the fluorescence decays of numerous single-Trp containing proteins have been examined. It was shown that, with the exception of a few proteins, all of them showed double or triple exponential decay behaviour [8]. The protein we examined, reconstituted $YidC_{W508}$, is no exception. The recovered lifetimes from the decay curve in Fig. 5A were 1,30 ns, 3,98 ns and 9,02 ns. After the addition of substrate no significant changes in the

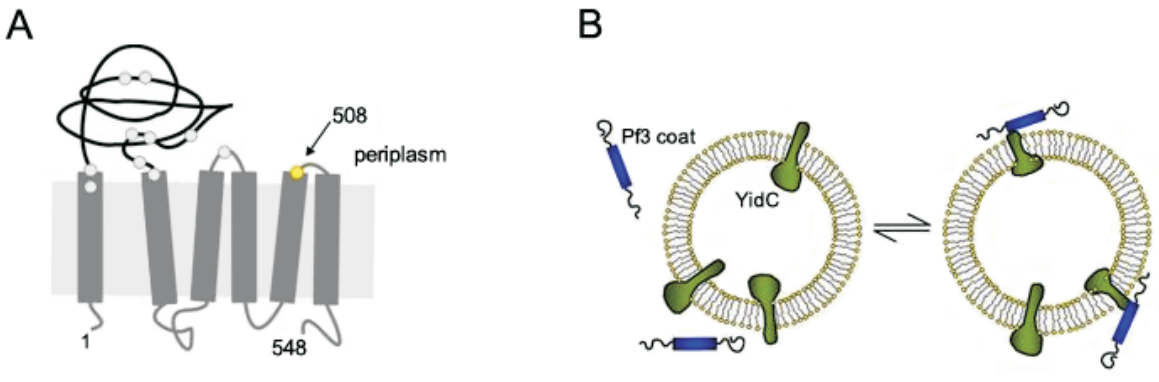

Fig. 4: (A) Proposed membrane topology of YidC. The approximate positions of the Trp residues along the structure are marked by circles. $YidC_{W508}$ has a single Trp residue at position 508 which is denoted by the arrow. (B) Artistic view of the reversible binding process of reconstituted YidC to Pf3 coat.

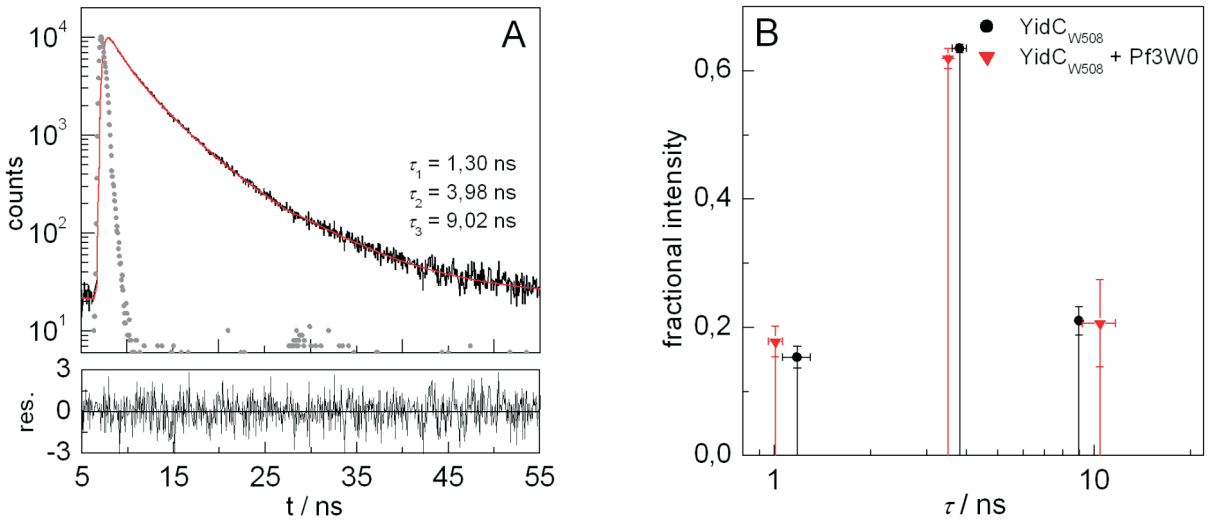

Fig. 5: (A) Fluorescence decay of reconstituted YidC$_{W508}$ recorded at "magic-angle" (54,7°) and the corresponding IRF (dotted curve). The best-fit values for the lifetimes are noted. (B) The mean values of the recovered lifetimes without and after the addition of the substrate Pf3W0 coat. All measurements were done at 25°C with a time resolution of 64 ps (in 20 mM Tris/HCl (pH 7.0), 300 mM NaCl and 5% isopropanol).

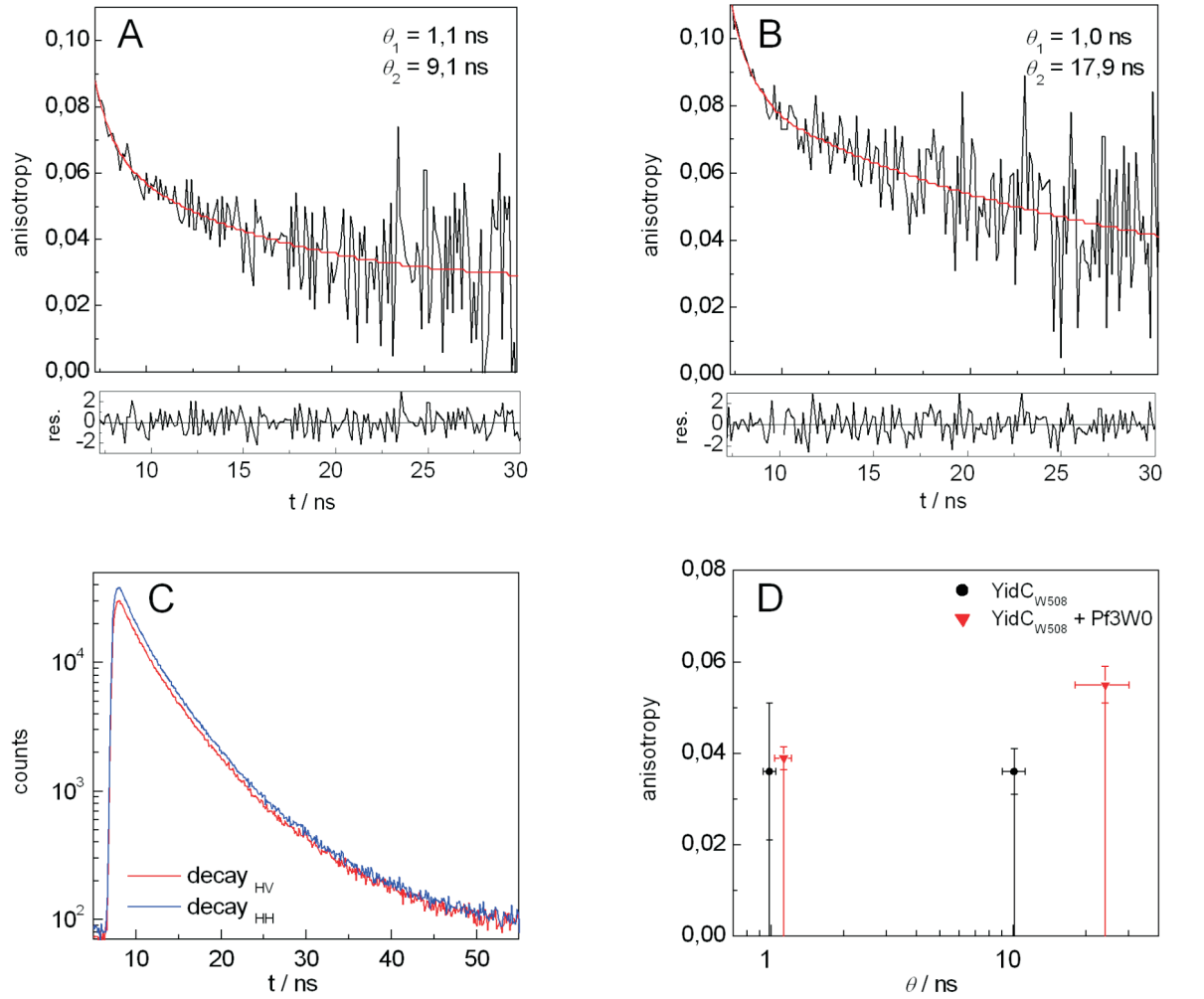

Fig. 6: Anisotropy decay of reconstituted YidCW508 (A) before and (B) after the addition of Pf3W0 coat protein. The best-fit values for the correlation times are noted. Intensity decay curves of YidC$_{W508}$, taken at vertical (VV) and horizontal (VH) emission polarizer, from which the decay curves in panel A is calculated. (C) Mean values of the recovered correlation times without and after the addition of the substrate Pf3W0 coat. All measurements were done at 25°C with a time resolution of 128 ps (in 20 mM Tris/HCl (pH 7.0), 300 mM NaCl and 5% isopropanol).

lifetimes were observed. The mean values for the recovered lifetimes of two independent experiments are shown in Fig. 5B.

In contrast, the fluorescence anisotropy decays showed relevant changes upon addition of the substrate. Whereas without substrate two distinct correlation times of around 1 ns and 10 ns were measured, after the addition of substrate the longer correlation time constant was nearly doubled in its value. The anisotropy decays with and without substrate as well as the mean values for the recovered correlation times of two independent experiments are shown in Fig. 6. These findings suggest that the protein surrounding at this certain single Trp residue undergoes a relevant conformational change upon binding of the insertase to its substrate.

It is obvious from Fig. 6A that the decay curves do not drop to a zero anisotropy value for long times (t). The reason for this is that the insertase is embedded in a lipid vesicle with a diameter of about 150 nm. By applying the Stokes-Einstein equation

$$\theta_{rot} = \frac{4\pi\ \eta\ R^3}{3kT}$$

which holds for globular particles, the rotational correlation time can be calculated with T = 298 K and η = 1 mPa s. The results for particles with diameters in the 100 nm range are presented in Tab. 2.

For vesicles of this size the rotational correlation times are in the millisecond range and thus cannot be resolved and the anisotropy decays for our samples show an constant off-set at t . To account for this large correlation times all calculations were made with a non-zero background value r_{inf}.

The zero-time anisotropy $r_0 = \Sigma r_i + r_{\text{inf}}$ (see eqn. 3) is a measure for the angular displacement of the absorption and emission dipole moment and is generally a function of the excitation wavelength [1]. In particular, the initial anisotropy for Trp shows a complex dependency on the excitation wavelength. This is caused by the existence of two, nearly perpendicular, transitions of the indole moiety S_0 ^1L_a and S_0 ^1L_b. With an excitation wavelength of 290 nm a value for r_0 0,12 is expected (see Fig. 7). The experimental values for r_0 (r_0 = 0,10 0,01 and r_0 = 0,11 0,01 before and after the addition of the substrate, respectively) are slightly lower than the expected value which can be caused by fast decay components with θ << 1 ns which cannot be recovered with this set up (IRF = 0,7 ns) as well as small misalignments of the polarizers.

Tab. 2: Rotational correlation times θ for spherical particle at T = 25° C

Diameter (nm)	100	150	200	300
θ (10^6 ns)	0,13	0,44	1,02	3,44

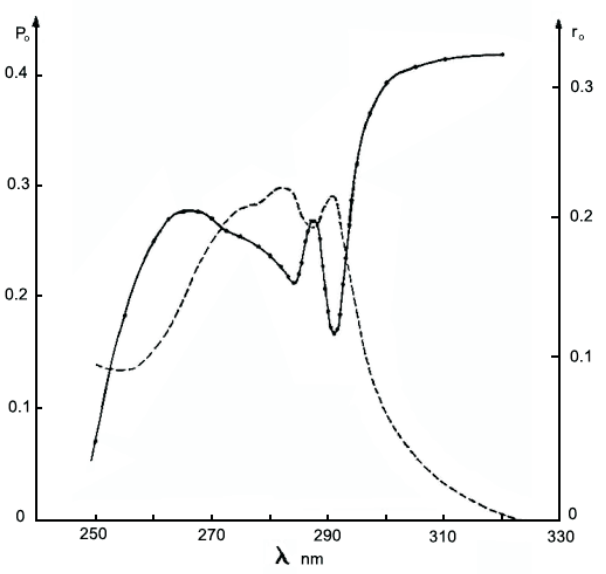

Fig. 7: Excitation spectrum (dashed line) and anisotropy spectrum (solid line) of tryptophan in propylene glycol at T = - 58°C. The initial anisotropy r_0 is denoted on the right ordinate (graph taken from [9]).

Summary and Conclusion

Our experimental data showed that it is possible to measure the dynamics of a single Trp residue in a protein with a FluoTime 100 lifetime spectrometer. Nevertheless, protein dynamics often take place on picosecond time scale [1] which cannot be reached with the described set-up due to the limited pulse width of the 290 nm pulsed LED. However, excitation sources with shorter pulse widths would allow to resolve even faster dynamics down to some tens of picoseconds using the FluoTime 100 spectrometer.

References

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Further information

1. Bibliography listing all publications with measurements based on PicoQuant instruments: http://www.picoquant.com/biblio.php

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