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In Vivo Synthesized Proteins with Monoexponential Fluorescence Decay Kinetics

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Tryptophan, when in a protein, typically shows multiexponential fluorescence decay kinetics. Complex kinetics prevents a straightforward interpretation of time-resolved fluorescence protein data, particularly in anisotropy studies or if the effect of a dynamic quencher or a resonance energy transfer acceptor is investigated. Here, a strategy is presented to overcome this limitation as time-resolved fluorescence data are presented of proteins containing a biosynthetically incorporated Trp analogue showing monoexponential decay kinetics.

Rationalization of the multiexponential decay kinetics of tryptophan (Trp)-containing proteins has been the subject of many papers.[1] Each lifetime (τ) corresponds to the reciprocal of the sum of radiative and nonradiative decay rates of a Trp population. Electron transfer (ET) between the excited Trp and the peptide backbone has been suggested as the major nonradiative decay route.[2] Recently, convincing data have been presented showing that different conformational states (rotamers) of Trp can account for the complex decay kinetics and the underlying process is variability of the ET rate to the peptide bond observed for different rotamers.[3] With this explanation, one has to conclude that a monoexponential protein fluorescence decay will only be observed in exceptional cases, that is, proteins with a Trp side chain fixed in one rotamer.

Chemical modification of Trp could yield a probe displaying photophysics less sensitive for ET to the peptide bond. To ensure a broad utility of such a probe, it must be possible to biosynthetically incorporate it in vivo using a Trp auxotroph expression host, as this strategy is much simpler than introducing the analogue via total chemical peptide synthesis or via chemically mis-acylated t-RNA technologies. To date, such a Trp analogue has not been identified.

We have recently reported a protocol for the efficient incorporation of Trp analogues (>95%), including 5-fluorotryptophan (5-FTrp), into single-Trp-containing mutants of the mannitol permease, EII^{mtl}, of *E. coli*.[4] Wild-type EII^{mtl} contains 4 Trp residues at positions 30, 42, 109, and 117, and single Trp mutants have been constructed, each with a Trp at the wild-type position (mutants W30, W42, W109, and W117).[5] 5-FTrp was incorporated into these mutants using an *E. coli* Trp auxotroph as described,[4] and the proteins were purified to homogeneity.[4] The absorption spectrum of 5-FTrp is slightly red-shifted as compared to Trp, allowing excitation up to 310 nm.[6] Interestingly, the fluorescence decay of W30, W42, and W117 was found to decay monoexponentially (Figure 1A−C). Fitting of the data with two exponentials did not significantly improve the quality of the fit as judged by visual inspection of the residuals, the χ^2 values, and the autocorrelation function of the residuals. An earlier study of these mutants, containing Trp, indicated that 3−4 exponentials were needed to properly fit the data.[5a] While the Trp in W30 and W42 are at a relative apolar and buried microenvironment, the Trp in W109 and W117 are at more polar and solvent-exposed positions.[5] Incorporation of 5-FTrp into four other single Trp-containing EII^{mtl} mutants (W97, W114, W188, and W327) also gave monoexponential fluorescence decays (Figure 1D−G). EII^{mtl} is dimeric under the conditions used, indicating that both 5-FTrp residues per dimer have equal or close to equal lifetimes. The only 5-FTrp-containing protein characterized so far not yielding a monoexponential decay is W109. The decay of this mutant could only be properly fitted with two exponentials ($\tau_1 = 5.14$ ns ($\alpha = 0.44$), $\tau_2 = 1.36$ ns). Note that position 109 is next to a cysteine (C110) and cysteine is known as a very efficient quencher of Trp fluorescence.[7] Most likely, C110 quenches 5-FTrp at 109, the multiexponential decay reflecting heterogeneity in the geometries between the 5-fluoroindole moiety and the sulfur atom of C110.[8]

The monoexponential decay of 5-FTrp might reflect the fact that the peptide bond is not a good electron acceptor for 5-FTrp. To investigate this, 5-fluoro-3-methylindole (5-F3MI) and *N*-acetyl-D,L-5-fluorotryptophanamide (5-FNATA) were synthesized, and their quantum yield (Φ) and τ were determined. The Φ values of 5-F3MI and 5-FNATA in neutral buffer at 20 °C are 0.25 and 0.21, respectively, using *N*-acetyl-tryptophanamide (NATA) as Φ standard. The reported Φ values of 3-methylindole (3MI) and NATA are 0.37 and 0.14, respectively,[2a] and ET to the amide bonds in NATA is almost exclusively responsible for this large difference in Φ.[2c,3] A similar trend is seen when the (monoexponential) lifetimes in neutral buffer at 20 °C of 5-F3MI ($\tau = 6.8$ ns) and 5-FNATA ($\tau = 4.4$ ns) are compared to those of 3MI ($\tau = 9.1$ ns) and NATA ($\tau = 3.0$ ns). These measurements strongly suggest that introduction of a fluoro atom at the 5-position of the indole moiety suppresses the ET rate to the peptide bond. More work is needed to estimate the sensitivity of 5-FTrp for the different quenching routes as found for Trp in proteins.[7]

5-FTrp is suitable for anisotropy studies as its intrinsic anisotropy (A_o) is 0.31 when excited at 310 nm,[9] a value similar to the maximum A_o value found for Trp.[1b] The monoexponential decay significantly simplifies the analysis of anisotropy decays as the uncertainty of fitting the data with an associated or nonassociated anisotropy decay model simplifies now to the latter model.[1b]

An interesting application of the monoexponential 5-FTrp fluorescence decay kinetics involves the discrimination of Trp residues in proteins containing several Trp's or oligomeric proteins, composed of single-Trp-containing subunits. The impact of a collisional quencher or resonance energy transfer (RET) acceptor on each 5-FTrp can be assigned in a time-resolved experiment. EII^{mtl} forms very stable dimers, and per dimer one mannitol binding site is present. A mannitol substrate analogue harboring a diazirinyl group as RET acceptor (azi-mannitol) was developed to localize the mannitol binding site, and RET was found in a steady-state

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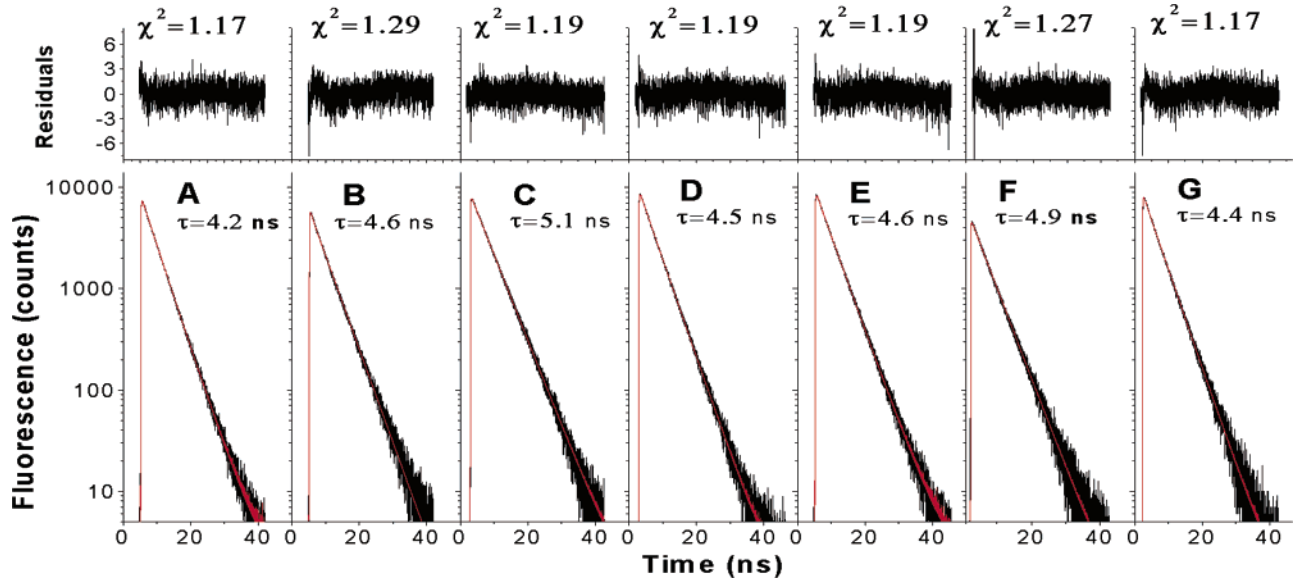

Figure 1. Fluorescence lifetime decays of W30 (A), W42 (B), W117 (C), W97 (D), W114 (E), W188 (F), and W327 (G) (black) and the corresponding monoexponential fit function (red). Conditions: 0.9−3.4 μM EII^{mtl} in 20 mM Tris-HCl pH 8.4, 250 mM NaCl, 0.5 mM reduced gluthatione, and 0.25% $C_{10}E_5$ detergent at 20 °C. Excitation was at 305 nm (W30 and W114 at 295 nm). The lifetime TCSPC measurements were performed with a frequency tripled modelocked Ti:Sapphire laser for excitation, delivering subpicosecond pulses of approximately 5 pJ at a 1.9 MHz repetition rate. The fluorescence was collected at 90° via a collimating lens, a calcite polarizer oriented at the magic angle, a WG320 filter, and a 350 nm interference filter with an 8 nm fwhm band-pass (OptoSigma) and was detected with a Hamamatsu R1564U-01 MCP. Data were collected during 5−10 min, and the samples were stable using these low excitation intensity conditions. The emission decay was stored in 4096 channels (12 ps/channel). Decays were corrected for equimolar concentrations of Trp-less EII^{mtl} [5], and *p*-terphenyl in ethanol was used as the monoexponential deconvolution reference (τ = 1.08 ns). Data were analyzed with a model of discrete exponentials using the TRFA data processing package, version 1.2, of SSTC, Belarusian State University, Belarus.

experiment with W30 and W42.[10] A time-resolved fluorescence experiment, using 5-FTrp-containing W30 and W42, allows the measurement of the individual distances. Mixing 5-FTrp-containing W30 with 1 mM azi-mannitol resulted in a biexponential fluorescence decay with lifetimes of 4.1 ns (α = 0.64) and 0.6 ns (see Supporting Information). Thus, one Trp in dimeric EII^{mtl} is not quenched by azi-mannitol, indicative that the distance is >17 Å, while the other Trp is quenched by 85%, corresponding with a distance <10 Å using a Förster distance of 10.4 Å.[10] Also, for W42 a biexponential decay was found in the presence of azi-mannitol, with one of the lifetimes essentially unchanged (τ = 5.0 ns, α = 0.47). The shorter τ of 2.9 ns corresponds to a distance of 11 Å. EII^{mtl} shows only mannitol binding affinity in its functional dimeric form.[5] Apparently, upon dimerization of EII^{mtl}, a new conformation exhibiting high affinity mannitol binding is formed, and the RET measurements show now that this binding site is not located symmetrically at the dimer interface.

In conclusion, work is presented showing that the fluorescence decay of 5-FTrp is monoexponential in seven out of eight proteins investigated. The multiexponential decay of one mutant is related to the presence of a neighboring cysteine residue. Although the disturbance of the monoexponential decay by an anomalous protein matrix cannot be excluded at the moment, our data suggest that monoexponential decay of 5-FTrp in a protein will be the rule rather than an exception. Pure samples and high 5-FTrp incorporation efficiency are essential, and probably these criteria have not been met in a previous study as a multiexponential decay was found for two 5-FTrp-containing proteins.[6a]

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Supporting Information Available: Fluorescence lifetime decays of W30 and W42 in the presence of azi-mannitol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) The phosphorescence emission of W109 was also found completely quenched in a study at ambient temperature, and this was attributed to the presence of C110.[5b]

(9) Conditions: 10 μM 5-FTrp in a glass of 20 mM sodium phosphate pH = 7.3/1,2 propylene glycol (10:90 (v/v)) at 173 K. The emission was passed via a 250 mm imaging monochromator and detected by an image-itensified CCD camera operated in single-photon counting mode.

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