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A subnanosecond resolving spectrofluorimeter for the analysis of protein fluorescence kinetics

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Summary

A spectrofluorometer is described consisting of an excitation source, optics, detector and time resolving electronics. The excitation source consists of a mode-locked Ar ion laser, which synchronously pumps a dye laser, followed by a frequency doubling device. The repetition frequency of the U.V. pulses (FWHM some ps) has been reduced by an extra-cavity electro-optical modulator. Provisions have been made in the optical configuration to determine both time-resolved fluorescence spectra and fluorescence anisotropy decay curves. The commercially available electronics have been optimized for maximum time resolution. The spectral output of the excitation source is confined between 280 and 310 nm, which encompasses the region for eliciting protein fluorescence. The performance of the complete system has been tested with single lifetime standards like *p*-terphenyl in cyclohexane or with *N*-acetyl-L-tryptophanamide in pH 7.5 buffer. Serum albumins from human and bovine sources have been employed as examples for time resolved fluorescence spectra and for the demonstration of anisotropy decay curves. Using these methods protein dynamics in the (sub)nanosecond time region can be directly explored.

Key words: mode-locked laser; synchronous pumping; frequency doubling; tryptophan; protein fluorescence; human serum albumin.

Introduction

The time dependence of protein fluorescence has been the subject of numerous investigations throughout the past decade (see e.g. Refs. 1–3). The strongest radiating components are tryptophanyl residues localized in different environments in the

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Abbreviations: FWHM, full width at half-maximum: CW, continuous wave; MCA, multichannel analyzer; TAC, time to amplitude converter; ADA, ammonium dihydrogen arsenate; ADC, analog to digital converter; PMT, photomultiplier; PTF, paraterphenyl; NATA, *N*-acetyl-L-tryptophanamide; HSA, human serum albumin; BSA, bovine serum albumin.

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protein matrix. The photophysical properties of this chromophore, like spectral distribution, emission maxima and fluorescence lifetimes, are very much dependent on environmental effects (reviewed by Lumry and Hershberger [4]). As a consequence of recently developed instrumental and computational improvements for measuring time-dependent fluorescence it turned out that tryptophan itself has interesting dynamic fluorescent properties, such as a distinct departure from exponential behaviour [5–8]. Rapid (sub)nanosecond relaxation processes monitoring tryptophan fluorescence were also investigated from analysis of exciplex fluorescence decay [9], oxygen quenching [10] and anisotropy decay [11].

This paper describes in detail the experimental set-up and protocol required to analyze the time dependence of protein fluorescence accurately. Fluorescence lifetime standards and globular proteins serve as illustrative examples.

Materials and Methods

p-Terphenyl (PTF, laser grade), obtained from Eastman Kodak, was dissolved in cyclohexane (fluorescent grade, Merck) and the solution was thoroughly degassed on a high vacuum line. *N*-Acetyl-L-tryptophanamide (NATA), purchased from Sigma, was dissolved in 0.05 M Tris-HCl buffer pH 7.5, made from distilled water. Solutions were freshly prepared prior to use. Human serum albumin (HSA) and bovine serum albumin (BSA) were obtained from Sigma. Solutions in 0.05 M Tris-HCl buffer pH 7.5 + 0.15 M NaCl were chromatographed on Sephadex-G100 (Pharmacia) columns prior to measurements. As a routine procedure solutions were always made with an absorbance = 0.2 at the excitation wavelength. Suprasil quartz 1 cm^2 fluorescence cuvettes were used throughout.

Routine procedures

Absorption spectra were recorded on a Cary 14 spectrophotometer. Fluorescence spectra were obtained with an Aminco SPF-500 fluorimeter. Steady-state fluorescence polarization was measured as described previously [12].

Description of experimental set-up

Fluorescence lifetimes, anisotropy decay and time-resolved emission spectra were measured with a set-up, schematically depicted in Fig. 1. Excitation came from the frequency doubled output of a synchronously pumped Rhodamine 6G dye laser, detection was performed with time-resolved single photon counting electronics. The system has been described in part in Refs. 13–15 for a non-synchronously pumped operation.

Our argon ion laser, a CR18UV from Coherent Radiation, was mode-locked using a brewster-cut acousto-optic prism from Harris Corporation. The mode-lock frequency (38 080 MHz) was stabilized by a digital synchronizer type TF 2173 from Marconi. Lasers, sample- and photomultiplier housings and optics were placed on a home-built table, consisting of marble plates on an air balloon-damped steel structure [14]. Home-built detector mounts for Telefunken BPW 28 avalanche

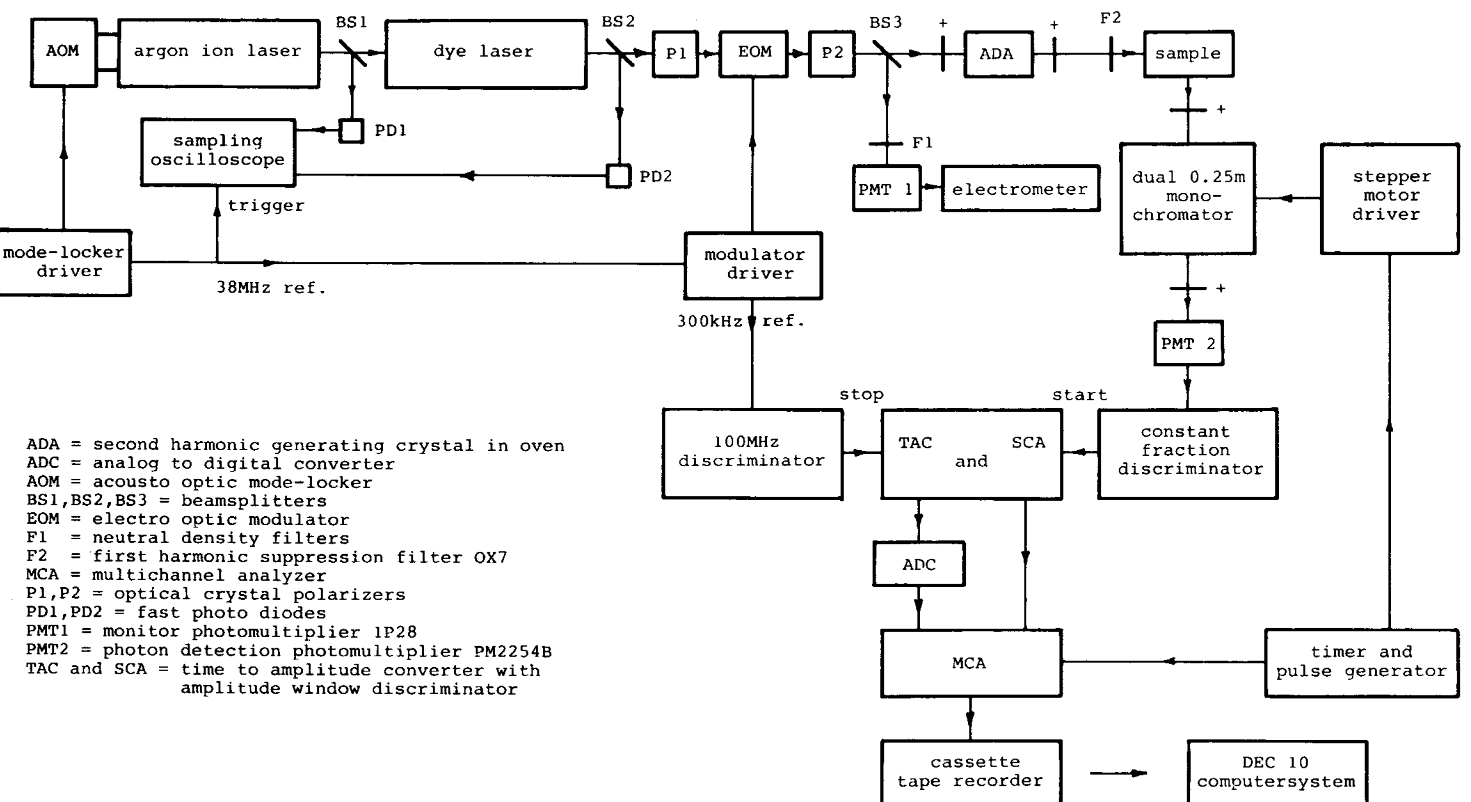

Fig. 1. Block diagram of experimental set-up.

photodiodes [16] were used for monitoring the optical pulse trains with a Hewlett Packard Sampling oscilloscope (modules 1430C, 1811 A and 182C). The cavity of the dye laser, a CR 590 from Coherent Radiation, was extended to match the cavity-length of the pump laser. For that purpose an invar rod (NIL036 from Wiggin Co.) was rigidly coupled to the original dye laser cavity. On the other end of the rod an alumina rail was mounted together with the new mount for the output mirror (Oriel rail 1142, carrier 1162, interferometric translation stage 1484, for precise tuning of the cavity length and high-precision adjustable mirror mount 1770 for angle tuning of the output mirror). The ratio of the lengths of invar and alumina bars was inversely proportional to the respective coefficients of linear expansion with temperature. In this way there was negligible displacement of the output mirror with respect to the other dye laser mirrors, when the ambient temperature varied within a few degrees.

For decreasing the rate of optical pulses from 76.160 MHz to 297.5 kHz (i.e. reduced by a factor of 256), an electro-optic modulator was used (model 22 from Coherent Associates) [14]. A standard side-window photomultiplier tube was used for monitoring the extinction ratio of the modulator. The light flux to the tube was regulated with reflecting neutral density filters, while linearity of the electro-meter reading (Keithly 610C) was checked. The extinction ratio, i.e. the ratio of the peak powers of suppressed and transmitted pulses at 588 nm, was about 1 : 400. We used the modulator in a single-pass arrangement [14], because after frequency doubling a much higher extinction ratio could be expected. This is due to the fact that with harmonic generation the output power is exponentially dependent on the input power.

The polarizer between dye laser and modulator was tuned to transmit the vertically polarized laser light. The polarizer behind the modulator was set exactly perpendicularly, transmitting horizontally polarized light and yielding vertically polarized UV light after frequency doubling. The reduced pulse train leaving the modulator was focussed into the doubling crystal using a fused silica thin lens with 10 cm focal length [17], the UV beam was recollimated using a similar lens. The doubling crystal was a 45° Z-cut ammonium dihydrogen arsenate cube (ADA) with 10 mm edges, placed in an oven with proportional temperature controller (Quantum Technology TC 10). The output beam from the crystal was filtered with an OX7 (Schott) first harmonic suppression filter and then deflected towards the sample housing, usually through a polarizing prism (Glan–Thompson prism) transmitting vertically polarized light.

The standard 10 mm light-path fluorescence cuvette holder was thermostated and the sample housing was flushed with dry air. The fluorescence was detected perpendicular to the excitation direction.

For time-resolved emission spectra two 0.25 m monochromators (Jarrell Ash 82-410, band width 10 nm) in tandem were used for scanning the fluorescence. The fluorescence was focussed on the entrance slit of the first monochromator through a sheet-type UV-polarizing filter (Polacoat), rotated at 54.7° with respect to the vertical position to minimize the effect of rotational motion on the lifetimes [18]. The wavelength stepping was controlled by a stepper motor-drive unit (Applied Photo-

physics). The synchronization with respect to the channel stepping of the multichannel analyzer (MCA, Laben 8001) was done by home-built electronics.

The fluorescence anisotropy decay measurements were carried out in a semi-simultaneous mode. The monochromator was replaced by a combination of a polarizer (Glan–Thompson prism) in a motorized mount and cut-off and/or band-pass filters (Schott WG 310, WG 360, UG 3; Balzers K36). The stepper motor-drive unit was then replaced by a rotator drive for the motorized mount of the polarizer. Upon receipt of a logic pulse from the timer of the MCA, the polarizer was rotated 90° from the original position. Two memory subgroups of the MCA were used to store the time spectrum of the parallel and perpendicular fluorescent components. During a period of 20 s (controlled by the timer of the MCA), the data of the parallel emission component were collected into one memory subgroup, then the polarization analyzer was rotated to the perpendicular position and during the next 20 s the perpendicular component of the fluorescence was detected and stored into the other memory subgroup. Finally, the analyzer was rotated back again and the whole procedure was repeated 20 to 40 times depending on the fluorescence yield.

The phomomultiplier detecting the emission photons was a Philips PM 2254, a S20 photocathode variant of the Philips XP2020, placed in a thermo-electric cooled housing from Products for Research (TE 104 RF with vacuum window optics). The single photon pulses from the anode of the PM 2254 were discriminated using a Canberra 1428 A constant fraction timing discriminator, delivering the start pulses for the time-to-amplitude converter (TAC). The stop pulses for the TAC came from a 100 MHz discriminator (Ortec model 436) discriminating the reference pulses from the modulator driver.

The TAC was a Canberra 2043, a converter with built-in single channel analyzer for selection of a time window in the spectrum displayed on the MCA. In principle, this selection can also be done with the controls for the analysis region in the analog to digital converter (ADC), but the TAC possesses a faster response [14]. Logic pulses are generated in the TAC, when a start–stop time difference falls within the window selected with the 'TIME' and Δ'TIME' controls. The MCA was then set in the multichannel scaling mode, the dwell time synchronized with the wavelength stepping of the monochromator. When measuring fluorescence or anisotropy decay the MCA was set in pulse height analysis mode, the ADC converting the analog pulses from the TAC. The digital output from the MCA was recorded on a cassette tape recorder (Racal Digideck) for storage. Data were evaluated on the university DEC-10 computer.

Starting-up procedure

Upon starting the experiments the laser tube discharge current was set at 50 A giving an output power of 5.5 W continuous wave (CW) at 514.5 nm and left at this current for at least 1 h for equilibration. Then, the mode-locking crystal was turned to optimum condition (temperature, frequency, R.F. power), resulting in an output of 1.9 W average mode-locked power. Some minutes were taken for equilibration. The pulse duration of the mode-locked light pulses was about 100 ps full width at half-maximum (FWHM) after correction for the rise-time of the detector [16].

With this pump power, the Rhodamine 6 G dye laser delivered about 620 mW average mode-locked power. A two-plate birefringent filter was inserted into the dye laser cavity for wavelength tuning. Using the relatively slow BPW 28 photodiodes it was possible to tune the synchronously pumped dye laser to optimum conditions (discussed in Ref. 19), in which the pulse duration is expected to be about 4 ps [20].

After achievement of optimum synchronous pumping the electro-optic modulator was aligned [14], followed by alignment of the doubling crystal. The temperature of the oven was set according to 90° phase matching at 590 nm and was kept at this temperature during all days of experiments. Every day minor adjustments were carried out to tune the wavelength of the dye laser for optimum doubling efficiency. Properly adjusted the complete system turned out to be stable for hours, so that reliable operation was warranted.

The UV output power of the ADA crystal could not be measured directly, since a low level power meter was not available. We checked it roughly using a standard side window photomultiplier and calibrated density filters and found at least 10 μW UV, more than sufficient for time-resolved single photon counting detection.

Applications

Single lifetime standards

In order to check the performance of the system, fluorescent compounds with a

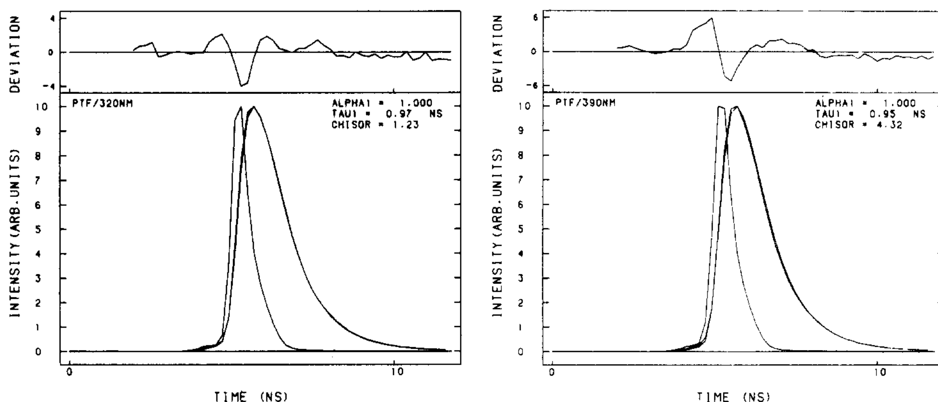

Fig. 2. Fluorescence decay of PTF in cyclohexane measured at 320 and 390 nm using 294 nm-excitation. The laser pulse, the experimental fluorescence response (F_{exp}) and fluorescence, convoluted with a single exponential (F_{calc}) are shown. Parameters are listed in the figure. The weighted residuals, $[F_{calc}(i)-F_{exp}(i)]/\sqrt{F_{exp}(i)}$, in each channel i (0.196 ns/channel) are shown in the upper panel. Pulse and fluorescence counts were collected until 20000 counts in the respective maxima. The time for collecting 20000 fluorescence counts in the peak channel depends on the lifetime and quantum yield of the fluorescence and amounted to about 1 min for PTF.

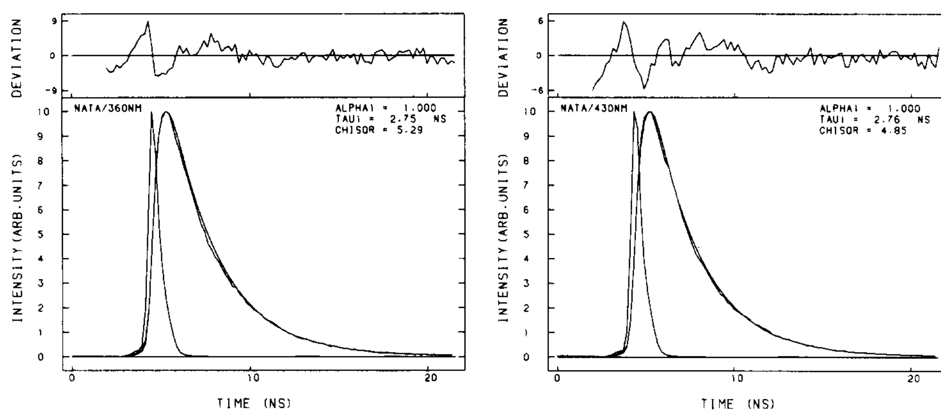

Fig. 3. Fluorescence decay of NATA in Tris-HCl at pH 7.5 measured at 360 and 430 nm upon 294 nm excitation. For further details see Fig. 2. It took about 5 min to collect 20000 counts in the peak channel of the NATA fluorescence response.

single fluorescence lifetime should be employed [21]. PTF in cyclohexane is a suitable standard for protein fluorescence, since its absorption and emission spectra overlaps with those of tryptophan [22]. Furthermore, PTF possesses a high quantum yield (near unity) and short fluorescence time constant (0.95 ns) [23,24]. We used this compound to check the wavelength dependence of the detection system within the range 320–400 nm. Fig. 2 shows an example of a decay analysis at two emission wavelengths. It was not necessary to introduce a significant time delay in the impulse response as required using other photomultipiers [24–26]. A nonlinear least-squares deconvolution procedure [27] provided the same lifetime as reported in the literature.

Another reference compound for protein fluorescence analysis is NATA. There has been much debate about the nonexponential fluorescent behaviour of L-tryptophan (see e.g. Ref. 8). The neutralisation of the ionizing residues seems to convert NATA into a normally behaving aromatic molecule as judged from the single exponential decay. We could reproduce and confirm the lifetimes across the whole emission band of NATA. Fig. 3 shows two decay analyses of NATA at two different emission wavelengths.

Time-resolved fluorescence spectra

Brand and Phillips and their coworkers have extensively discussed the measurements of time resolved emission spectra [28,29]. Brand constructed 3-dimensional graphs (fluorescence versus time versus wavelength) from decay analysis at different emission wavelengths. Phillips et al. discussed both ways for recording those graphs and illustrated this by using a frequency-doubled Ar ion laser for excitation.

We have scanned spectra at discrete time intervals after the laser pulse. Both the time interval and the gate width could be adjusted. In order to observe relative time-dependent shifts in emission spectra it is appropriate to compare peak-normalized fluorescence spectra at different time intervals after the laser pulse. As test

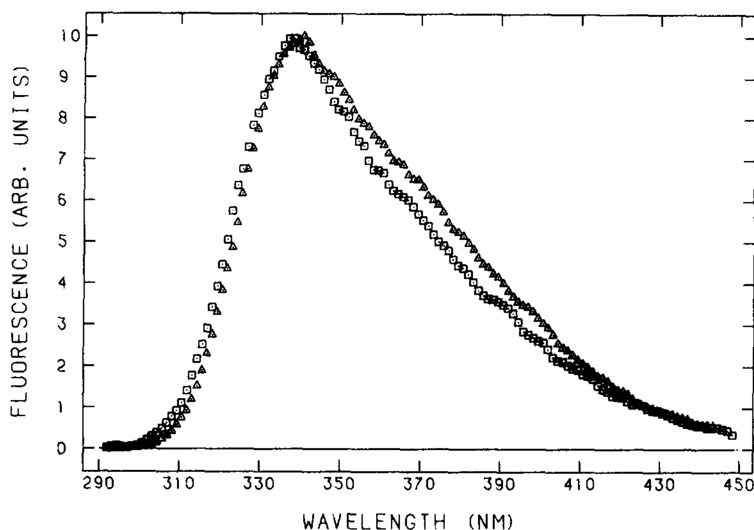

Fig. 4. Peak-normalized fluorescence spectra of HSA at 2°C taken at time intervals of 1.3 ns (□□□) and 19 ns (△△△) after the laser pulse maximum (λ_{exc} = 294 nm). The gate width for the spectrum, taken at early time, was 1.3 ns, for the other spectrum it was 10 ns. The peak channel of the first spectrum (□□□, scan time 30 min) contained 1435 counts, for the other spectrum (△△△, scan time 80 min) there were 917 counts in the peak.

example we used HSA, which is an interesting globular protein since it contains one single tryptophanyl residue. In Fig. 4 peak-normalized spectra taken at 1.3 ns and at 19 ns after the excitation pulse are shown. The spectra are uncorrected for wavelength dependence of both the photomultiplier sensitivity and the monochromator efficiency. The two spectra cannot be superimposed and are evidence that more emitting species are present. Parameters describing HSA fluorescence decay are

TABLE 1

BIEXPONENTIAL FLUORESCENCE DECAY PARAMETERS FOR HUMAN SERUM ALBUMIN AT 20°C

Wavelength (nm)	a_1	τ_1 (ns)	a_2	τ_2 (ns)	$<\tau>$ [a] (ns)
320	0.54	2.8	0.46	6.6	4.5
340	0.56	3.6	0.44	7.3	5.2
360	0.64	4.2	0.36	7.9	5.6
380	0.60	4.1	0.40	8.0	5.7
400	0.65	4.3	0.35	8.4	5.8
420	0.63	3.9	0.37	8.3	5.5

[a] $<\tau> = \sum_{i=1}^{2} a_i \tau_i, \ (\sum_{i=1}^{2} a_i = 1).$

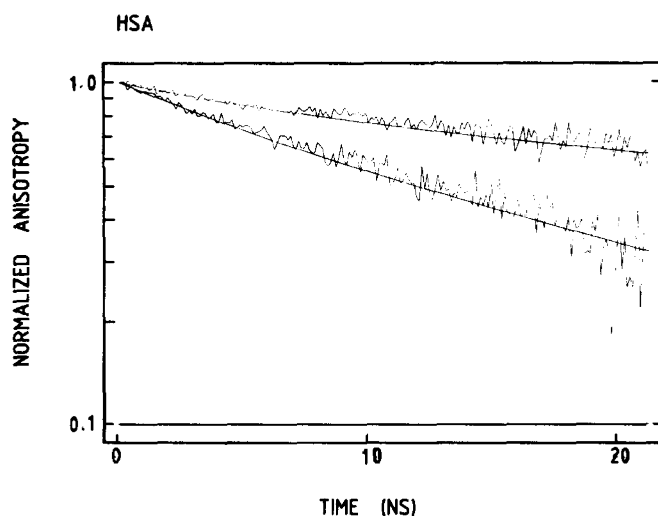

Fig. 5. Fluorescence anisotropy decay of HSA taken at 2°C and at 31°C. Decay parameters are given in Table 2.

collected in Table 1. The fluorescence decay of HSA is not a single exponential. Our data are in accordance with those of Wahl and Auchet [30].

Decay of protein fluorescence anisotropy

This type of measurement has been described by Munro et al. [11] for a wide range of single tryptophan containing proteins. These authors used monochromatic synchroton radiation as source of excitation and single photon counting in the

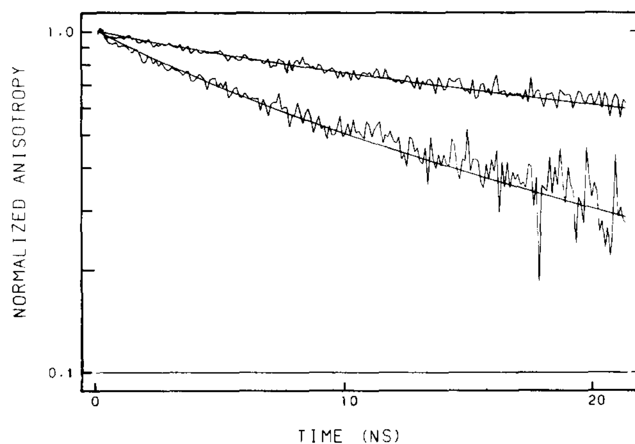

Fig. 6. Fluorescence anisotropy decay of BSA taken at 2°C and at 31°C. Decay parameters are given in Table 2.

TABLE 2

ANISOTROPY DECAY PARAMETERS FOR HSA AND BSA AT DIFFERENT TEMPERATURES [a]

T	HSA	BSA	Calculated [b]
2°C	$\alpha_1 = 0.15$	$\alpha_1 = 0.13$	
	$\phi_1 = 66$ ns	$\phi_1 = 62$ ns	$\phi = 46$ ns
	$\alpha_2 = 0.03$	$\alpha_2 = 0.03$	
	$\phi_2 = 6$ ns	$\phi_2 = 9$ ns	
	$r_0 = 0.18$	$r_0 = 0.16$	
21°C	$\alpha_1 = 0.13$	$\alpha_1 = 0.11$	
	$\phi_1 = 33$ ns	$\phi_1 = 34$ ns	$\phi = 25$ ns
	$\alpha_2 = 0.03$	$\alpha_2 = 0.03$	
	$\phi_2 = 6$ ns	$\phi_2 = 6$ ns	
	$r_0 = 0.16$	$r_0 = 0.14$	
31°C	$\alpha_1 = 0.12$	$\alpha_1 = 0.09$	
	$\phi_1 = 23$ ns	$\phi_1 = 25$ ns	$\phi = 20$ ns
	$\alpha_2 = 0.03$	$\alpha_2 = 0.05$	
	$\phi_2 = 6$ ns	$\phi_2 = 6$ ns	
	$r_0 = 0.15$	$r_0 = 0.14$	

[a] $\phi_{1,2}$: rotational correlation times; $r_0 = \alpha_1 + \alpha_2$: anisotropy at $t = 0$.

[b] With the formula $\phi = M_r(\bar{v} + h)\eta/RT$, M_r = molecular weight (= 68000), $\bar{v}$ = specific volume (≈ 0.735 cm^3/g), h = hydratation factor (≈ 0.2 cm^3/g), η = viscosity (cp), R = gas constant and T = temperature (K).

detection channel. We have reproduced measurements on HSA using our laser excitation source and extended the experiments to BSA, a globular protein containing two tryptophans. Our results are displayed in Figs. 5 and 6 for HSA and BSA at two different temperatures. The correlation times have been collected in Table 2, in which we also incorporated values calculated from an empirical formula. The accordance between calculated and observed data is rather good and illustrated that multitryptophan containing proteins can be examined without any problem. Noteworthy is the small initial anisotropy (Table 2) due to excitation into other electronic states [31]. The results also clearly indicate that the tryptophan residues mainly rotate with the protein as a whole.

Discussion

With the instrumentation described we were able to demonstrate all facets of time-dependent protein fluorescence. Fluorescence decay at different emission wavelengths does not encounter any difficulties. Time-resolved emission spectra can be directly measured at short time intervals after the laser pulse (FWHM 0.5 ns with single photon counting detection) and do not need to be reconstructed from decay profiles. Anisotropy decay can also be accurately determined and can be analyzed directly. The excitation source is a good counterpart of that available from synchro-

tron radiation [11]. Nonetheless, despite the good time characteristics of both excitation sources, the time resolving power is limited by the detector. We can therefore state that subnanosecond relaxation processes can be registered with the described set-up. Upconversion techniques as described by Beddard et al. [32] provide an alternative to investigate picosecond relaxation. However, the gain in time resolution is acquired at the cost of sensitivity as delivered by the single photon counting technique.

Simplified description of the method and its applications

A complete description of a time-resolving spectrofluorimeter appropriate for protein fluorescence studies has been given. The system consists of a mode-locked Ar ion laser which synchronously pumps a Rhodamine 6G dye laser, as source of excitation, and a fast photomultiplier with single photon counting electronics in the detection channel. The repetition frequency of the excitation pulses (76 160 MHz, FWHM some ps) has been reduced to 297.5 kHz by passing the beam through an electro-optical modulator. Frequency doubling was accomplished by means of an ADA crystal for second harmonic generation. The measured FWHM of the UV pulses is around 0.5 ns depending on the configuration like double monochromator or filters in the detection light path. The broadening of the pulse is mainly due to transit time variations in the PMT. We have tested the system with single-lifetime standards like PTF in cyclohexane and NATA in aqueous buffer using a fixed excitation wavelength and different emission wavelengths. Human serum albumin was used as an example to obtain time-resolved fluorescence spectra at distinct times after the laser pulse. It was demonstrated that these spectra could be obtained without deconvolution from the excitation pulse. Anisotropy decay measurements were also carried out, and rotational correlation times were obtained with high precision. Using the described set-up the dynamic properties of proteins can be successfully explored.

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