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PICOSECOND RESOLUTION OF OXYTOCIN TYROSYL FLUORESCENCE BY 2 GHz FREQUENCY-DOMAIN FLUOROMETRY

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The technique of frequency-domain fluorometry has been extended to 2000 MHz using the harmonic content of a picosecond laser source and a microchannel plate photomultiplier tube. This new instrument was used to resolve complex subnanosecond intensity and anisotropy decays of the tyrosyl emission of oxytocin. The intensity decay was found to contain at least three exponential components, 80, 359 and 927 ps. The anisotropy analysis revealed a 29 ps torsional motion of the tyrosine residue as well as a 454 ps overall rotational correlation time. The time resolution of this method should permit the comparison of experimental results with theoretical models for motions of proteins.

1. Introduction

There is considerable interest in the dynamics of proteins, such as local torsional motions of individual amino acid residues and overall rotational diffusion [1–3]. Experimental determination of the former is of interest for comparison with molecular dynamics calculations, and the latter to obtain the hydrodynamic shapes of proteins. Tyrosine and tryptophan are intrinsic fluorescence probes in proteins. This fact, and the nanosecond time scale of fluorescence emission, have resulted in the expectation of detailed information on protein dynamics and hydrodynamics.

To date, the time resolution of fluorescence spectroscopy has been limited by the pulse widths of flash lamps, the expense of laser sources and the timing limitations of photomultiplier tubes [4]. In an attempt to improve the resolution of complex decay phenomena we and others [5,6] developed an alternative to time-domain measurements. The frequency-domain method was shown to provide good resolution of multiexponential intensity and anisotropy decays [7–9]. The resolution of rapid and/or complex decays is limited by the upper frequency limit of the measurements. In this report we extend the frequency range 10-fold over all the previous measurements. The present frequency range of 10–2000 MHz was found to provide good resolution of the complex intensity decay and rotational kinetics of oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2).

2. Materials and methods

Frequency-domain data were obtained on the instrument described previously [5], with the following modifications: (1) The light source was a 3.79 MHz train of 5 ps pulses from a cavity-dumped dye laser, rhodamine 6G, frequency-doubled to 287 nm. (2) The output of a 500 MHz frequency synthesizer was multiplied 2- or 4-fold. (3) The standard photomultiplier tube was replaced with a microchannel plate photomultiplier tube (Hammamatsu R1564), which was externally

cross-correlated. These technical features will be published elsewhere. The frequency-dependent data were analyzed using methods previously described in detail [5,8,10].

Oxytocin was obtained from Sigma and used without further purification. Its emission spectrum was characteristic of tyrosine. The experiments were performed in 0.05 M phosphate (pH 7.0) at 25°C, using 287 nm excitation. The emission was observed through a 300 nm interference filter (10 nm bandpass). Examination of buffer alone indicated that background fluorescence and/or scattered light contributed less than 0.5% to the measured emission.

3. Results and discussion

Frequency-domain data for the intensity decay analysis are shown in fig. 1. The data extend from 11.38 MHz to 1.91 GHz and include 27 frequencies. The data were easily adequate to determine that at least three exponential components are needed to account for the data. The dashed line on fig. 1 (top) shows large deviations from the single-exponential model. These deviations and the extreme value of $\chi^2_R = 377$ (table 1) are easily adequate to reject this model. Even the two-component decay can be rejected with reasonable certainty, based on the 3-fold decrease in χ^2_R for the three-component fit and our approx. 50 degrees of freedom [11]. The most remarkable feature of these data is that the information content and signal-to-noise ratio permit facile resolution of two- and three-component tyrosyl decays. We know of no other multicomponent tyrosyl resolution on the subnanosecond time scale. Libertini and Small [12] used a mode-locked laser source and time-correlated photon counting to recover three decay times ranging from 1 to 4 ns for the single tyrosine residue in a histone H1.

Data to recover the anisotropy decay kinetics are shown in fig. 2. These data are the phase angle

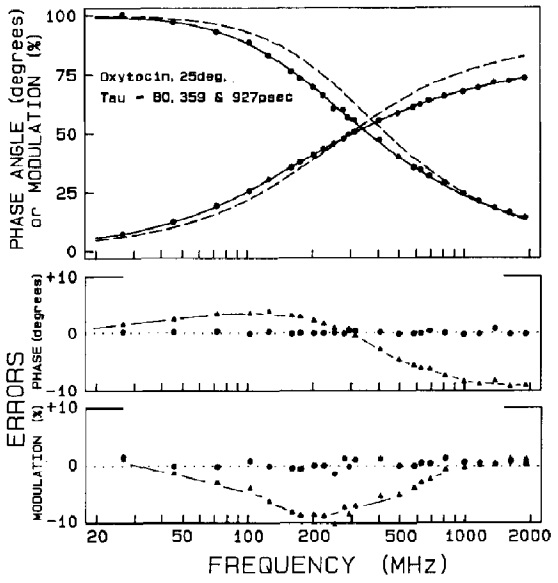

Fig. 1. Frequency-domain data for the tyrosine intensity decay of oxytocin. The upper panel shows the data (●). The solid line is the best three-exponential fit, and the dashed line the best one-exponential fit. The lower panels show the deviations between the data and the calculated values for the one (▲) and three (●) decay time models.

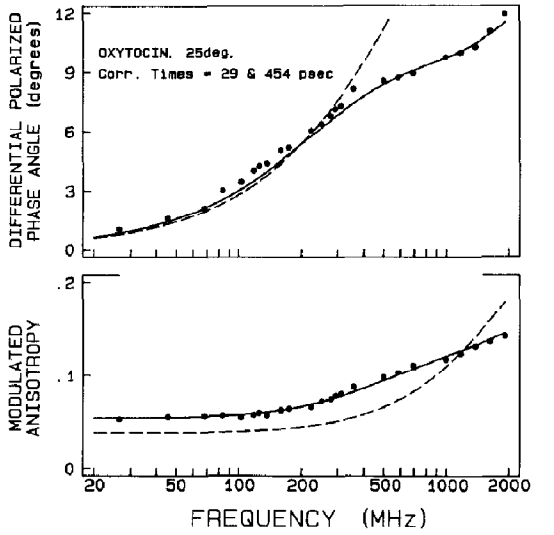

Fig. 2. Frequency-domain data for the tyrosine anisotropy decay of oxytocin. The data (●) could not be fitted using a single correlation time (------), but were adequately fitted, using two correlation times (———).

Table 1

Intensity and anisotropy decay parameters of the tyrosine emission from oxytocin

Decay type					
Intensity	n [a]	τ_i (ns)	α_i	f_i	χ^2_R
	1	0.634	1.0	1.0	377.0
	2	0.140	0.42	0.11	
		0.830	0.58	0.89	5.9
	3	0.080	0.29	0.04	
		0.359	0.27	0.19	
		0.927	0.43	0.77	2.1
Anisotropy	n	θ_i (ns)	$r_0 g_i$ [b]	χ^2_R	
	1	0.083	0.320	292.0	
	2	0.029	0.208		
		0.454	0.112	3.3	
	3	0.028	0.105		
		0.029	0.104		
		0.454	0.112	3.4	

[a] Number of exponential components.
[b] $\Sigma r_0 g_i = 0.32$, and held fixed at this value.

difference between the polarized components of the emission (top) and the frequency-dependent anisotropy (bottom) [13]. Attempts to fit the data with a single rotational correlation time (- - - - -) result in a completely unacceptable match to the data (fig. 2). In contrast, a two-correlation-time model with values of 29 and 454 ps results in a good fit (———). The 29 ps correlation time accounts for 60% of the total anisotropy decay, and is probably due to segmental motions of the phenol ring independent of overall rotational diffusion. The 454 ps correlation time is comparable to that expected for overall rotational diffusion of oxytocin in water, and agrees with an earlier estimate based on oxygen quenching [14]. The use of three correlation times did not improve the fit, as evidenced by the similar value of χ^2_R and nearly identical values for the correlation times and their associated amplitudes (table 1). Note that the first two components combined are the same as the first component in the two-correlation-time fit. It is important to note that the measurements to 2 GHz provide considerable information content above the data to the previous 200 MHz limit. Data to 200 MHz would not display the shoulder seen at 600 MHz, which represents the transition from rotational diffusion to segmental motions.

4. Conclusion

Frequency-domain data to 2 GHz provide unambiguous resolution of multiexponential tyrosyl intensity and anisotropy decays. Such data can be compared with theories of protein motion and diffusion.

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