Solvation in protein (un)folding of melittin tetramer–monomer transition

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Protein structural integrity and flexibility are intimately tied to solvation. Here, we examine the effect that changes in bulk and local solvent properties have on protein structure and stability. We observe the change in solvation of an unfolding of the protein model, melittin, in the presence of a denaturant, trifluoroethanol. The peptide system displays a well defined transition in that the tetramer unfolds without disrupting the secondary or tertiary structure. In the absence of local structural perturbation, we are able to reveal exclusively the role of solvation dynamics in protein structure stabilization and the (un)folding pathway. A sudden retardation in solvent dynamics, which is coupled to the change in protein structure, is observed at a critical trifluoroethanol concentration. The large amplitude conformational changes are regulated by the local solvent hydrophobicity and bulk solvent viscosity.

fluorescence spectroscopy | preferential solvation | protein folding | ultrafast hydration

Proteins need to assemble themselves, or “fold,” forming a complex 3-dimensional structure to carry out their appropriate functions (1). In contrast, when proteins fold incorrectly (misfold), there can be serious consequences, e.g., Alzheimer’s disease and cancer (2). The process of protein folding is an inherently complex process along an energy landscape that involves not only the possible conformations and segmental motions of the protein, but also the interactions with surrounding solvent molecules (water in most cases) (3–5). The dynamics of solvent motions directly affects protein conformational transitions. The role of solvation in the dynamic balance between structural integrity and conformational mobility is a key feature in protein function (6). Thus, the elucidation of solvation dynamics on the involved time scales, from ultrafast local solvent motion to slow, large amplitude protein-associated motion, is essential to the understanding of protein structure and functions. To systematically examine the relationship of solvent motion to protein folding, we studied the solvation dynamics of a model peptide, melittin, which possesses a unique structural transition from the native folded tetramer to a helical monomer, both of which contain the same secondary and tertiary structure.

Melittin is a 26 residue peptide, which is the major component of honeybee venom. At low peptide and ionic concentration and neutral pH, melittin is in a random coil configuration. The α -helical tetramer conformation (Fig. 1) can be induced at alkaline pH, high salt, high acid, or high peptide concentrations (7). The folding of the tetramer is often used as a model for protein folding. Under physiological conditions melittin is found to be in the random coil state and transforms into a monomeric α -helix upon binding with the cell membrane, where it lyses the cell membrane. The helical monomer consists of 2 helical segments separated by a coiled region containing a proline residue (8–11). In the melittin tetramer, the intrinsic solvation probe of tryptophan (Trp) is located at the junction between 2 α -helical monomers and is partially buried. However, Trp is fully exposed to the solvent environ-

ment in the helical monomer. The α -helical monomer configuration has also been induced for micellar systems (12) and with the presence of alcohol cosolvents (Fig. 1) (13, 14).

In this study, we use the cosolvent 2,2,2-trifluoroethanol (TFE) in water to denature the native folded state of melittin and stabilize the monomeric α -helix (Fig. 1). A variety of mechanisms have been proposed to account for the conformational changes due to the presence of fluorinated cosolvents, i.e., a lowering of dielectric constant in aqueous solutions (15), increase in intramolecular hydrogen bonding (16), enthalpic and entropic effects (17, 18), preferential binding of fluoroalcohols to the protein (19–22), and cluster formation resulting in micellar or clathrate-like structures (15). In this report, large-scale conformational changes assisted by the solvent lead to a transient structure of the tetramer, thus the transition is not a 2-state process. The sensitivity of Trp to local solvent viscosity allowed for the measurement of the extent of preferential solvation. The stable helical conformation of melittin monomer in aqueous and alcohol solution permits the observation of changes due to local solvent dynamics around the Trp probe caused solely by the tetramer assembly without the need for sequence mutation. By avoiding changes in sequence and secondary structure, we are able to isolate changes in solvation, thereby investigating the subtle interplay between local and bulk solvent dynamics and protein structural integrity. This critical role of the solvent is consistent with the model presented by Frauenfelder and colleagues (5).

Results

Melittin Structure and Steady-State Spectra. The experimental conditions were designed such that melittin would be in the tetramer configuration in a neat buffer solution, and unfolds to 4 helical monomers with the addition of TFE cosolvent. The α -helical secondary structure of melittin was maintained for all cosolvent concentrations, as confirmed using circular dichroism (Fig. 2A). Small additions of TFE cosolvent increase the helical content slightly, as shown in Fig. 3. The unfolding of the melittin tetramer can be monitored using steady-state fluorescence emission. It is well established that the steady-state fluorescence spectrum of Trp mainly depends on the extent of its exposure to polar solvents. There is a large red-shift in the emission spectra of Trp (see Fig. 3) with the addition of TFE, indicating an increase in its exposure to solvent. We observed very little change in the position and shape of the steady-state absorption spectra with the addition of TFE. The circular dichroism and fluorescence spectral changes are indicative of a relatively stable secondary and tertiary structure, but a changing quaternary structure. The addition of alcohol cosol-

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Tetramer to Monomer Transition

Fig. 1. Melittin tetramer–monomer ($\times 4$) transition. Melittin structure in the native tetramer (*Left*), each chain is highlighted with a different color. The tryptophan residues are highlighted in orange. Melittin structure in the α -helical monomer configuration (*Right*). The structure is shown both as a surface (*Upper*) and ribbon (*Lower*; tetramer is shown at a different angle) representation to better demonstrate burial of the tryptophan residues and structure of the helical backbone in both phases.

vent thus results in the breakup of the tetramer in favor of the α -helical monomer. The α -helical secondary structure remains stable for intermediate (10–75%) TFE contents and decreases for 100% TFE. The decrease in helicity demonstrated for melittin in pure TFE may be representative of an increase in flexibility between the 2 α -helical segments (23).

Fig. 2. Secondary structure and solvation dynamics. Results for 1 mM melittin with various cosolvent compositions. (A) Circular dichroism spectra for melittin with 0, 10, 40, and 100% (vol/vol) TFE. (B) Solvation energy changes for 1 mM melittin in 0, 12, 50, and 100% (vol/vol) TFE and bare Trp in neat buffer and TFE.

Fig. 3. Helicity, fluorescence energy shift, local solvent dynamics, and viscosity. Fluorescence emission maximum (squares), helical structure content (circles), the rate of local solvent motion, $1/\tau_2$ from Table 2 (triangles) and estimated rate of solvent motion, $1/\tau_2^*$ (dotted line), and the viscosity of water-TFE mixtures adapted from ref. 24 (diamonds).

Time-Resolved Fluorescence Dynamics. Time-resolved fluorescence techniques were used to measure the rotational motion of the peptide and the solvation dynamics. Representative anisotropy data are shown in Fig. 4 and a summary of the measurements are given in Table 1. The rotational anisotropy of a molecule depends on the viscosity of the solvent and the shape of the solute (see, e.g., ref. 24), and was used to monitor the tetramer to monomer transition in this study. The viscosity of water-TFE solutions doubles from 0 to 50% (vol/vol) TFE, and varies between 1.6 and 1.8 mPa·s for TFE contents >50% (25). For all cosolvent content, we observed a fast initial depolarization of ≈ 100 fs due to the internal conversion between $^1\text{L}_a$ and $^1\text{L}_b$ states of Trp (6, 26). The results (Table 1) indicate a large reduction in overall macromolecular tumbling time (T_2) from 3,200 to 1,020 ps with addition of TFE, and rotational time reaches a maximum for 12%-TFE solution; this represents up to a 30% increase in the hydrodynamic volume of the solute. The anisotropy results are in qualitative agreement with previous measurements made for melittin in buffer solution (27, 28). The reduction in tumbling time is consistent with a change in tertiary structure from the melittin tetramer to the helical monomer.

Time-resolved fluorescence measurements were made for melittin in 6 cosolvent concentrations. Nine emission wavelengths, spanning the fluorescence spectra, were measured for each cosolvent content. A global fitting strategy was used to fit the transients. The lifetime of Trp in the melittin systems was determined by fitting time-correlated single photon counting (TCSPC) data. Because of the symmetry of the melittin tetramer, the 4 Trp residues have nearly identical solvation environments (29). The data show 2 lifetime components

Fig. 4. Dynamic rotational anisotropy. Results for 1 mM melittin; TFE contents and deduced time constants are given in each panel. (Inset) Solvation of the peptide at that concentration.

representing the 2 major rotational conformers of Trp in peptide systems (30). The lifetime values varied from 0.69 to 1.13 ns and 2.78–2.96 ns for different concentrations of TFE.[†]

The lifetimes were longest for low TFE concentrations, which can be indicative of the burial of Trp in the hydrophobic core of the melittin tetramer. The lifetime values determined by the global fitting of TCSPC data were incorporated into the fitting of the fluorescence up-conversion data. Because of the considerable differences of the time constants, these data were typically fit to 4 to 5 discrete exponential functions, two of which are the lifetimes of Trp in the peptide system. The remaining time constants represent the solvation dynamics of the system and are strongly dependent on the wavelength monitored.

Construction of Solvation Energy of Relaxation. The decay observed at blue wavelengths and corresponding rise at red wavelengths are the signature of solvent relaxation. The time-resolved emission spectra are constructed from the femtosecond transients, as described in ref. 31. The spectra are then fit to a log-normal function and the overall emission maxima $\bar{\nu}_S$ (Stokes shift) and lifetime-associated maxima $\bar{\nu}_1$ are extracted. The solvation energy of relaxation functions $\Delta E_S(t)$ are constructed using the equation:

[†]Specific interactions between TFE and indole, the chromophoric moiety of Trp, have been suggested to occur via, e.g., OH(TFE)··· π (Trp) H-bonding (30, 31). Our measurements of the steady-state and time-resolved fluorescence of bare Trp in neat TFE also indicate the presence of specific interactions between Trp and TFE (see SI). For melittin, however, we could not find any strong signature of the specific interactions; such interactions are often accompanied by steady-state spectral and fluorescence lifetime changes. The changes in the steady-state emission spectra and fluorescence lifetime with the increase of TFE concentration follow the conformational changes of the protein quaternary structure alone. It should be noted that 1,1,1,3,3,3-hexafluoroisopropanol, an even stronger helix inducing agent than TFE, is reported to have only a diffusive role in the solvation of melittin, without any evidence of specific interactions (22). The amphiphilic nature of TFE in binary mixtures and the heterogeneous surface morphology of the peptide are inferred to hamper the specific interactions.

Table 1. Anisotropy measurements of melittin in different cosolvent concentrations

% (v/v) TFE	T_1 , ps	T_2 , ps	θ^*
0	66.1	3200	21.6
8	59.2	3200	20.6
12	64.1	4300	23.2
25	67.0	2800	27.6
50	29.4	1600	22.8
100	11.9	1020	21.6

*The cone semi-angle of local Trp wobbling motion; see ref. 44.

$$\Delta E_S(t) = \bar{\nu}_S(t) - \bar{\nu}_1(t).$$

The solvation energy changes are shown for 4 representative cosolvent compositions in Fig. 2B. A summary of all of the results are given in Table 2. The ultrafast time scale (τ_1), ≈ 1 ps, represents fast librational and rotational motions of bulk solvent molecules. The intermediate time scale (τ_2), ≈ 4 –10 ps, is attributed to the rotational and translational motions of the local solvent molecules. The longest time scale ≈ 100 ps (τ_3) is related to the collective motion of bound solvent molecules and their exchange with the bulk ones; this process is strongly coupled to peptide motions (31). The results in Table 2 are best viewed in terms of changes in solvent motions around a constant conformation. From our previous measurements of steady-state fluorescence spectra, circular dichroism, and rotational anisotropy, we can conclude that melittin with both 0 and 8% (vol/vol) TFE content is in the tetramer configuration. For small additions of TFE we observed retardation of the solvation dynamics for the two fastest time constants. It is interesting to note that the peptide in 25 and 50% TFE mixtures display the same solvation dynamics at all time scales. The retardation and stabilization of local solvation dynamics are evidence of the preferential solvation of the peptide by fluorinated alcohols, as will be further discussed in the next section. For TFE content greater than $\approx 20\%$, melittin is in the monomeric α -helix state according to our spectroscopic and dynamics data. The solvation dynamics around Trp in the monomeric state is significantly retarded compared with that of the native tetramer, which is reflective of a significant increase in solvent viscosity (see below).

The above results can be summarized as follows. First, with TFE content between 0 and 8%, melittin exists as a tetramer. Whereas it adopts an α -helical monomeric conformation with TFE contents $>20\%$, this is evident from circular dichroism, fluorescence, and depolarization dynamics measurements. Second, solvation-energy relaxation dynamics is represented by 3 distinctive time scales of ≈ 1 ps (τ_1), 4–10 ps (τ_2), and ≈ 100 ps (τ_3), which correspond to librational and rotational motion of free solvent molecules, local bound solvent motion, and collective solvent dynamics coupled to the segmental motion of the peptide, respectively. Third, fast solvation time constants of τ_1 and τ_2 retard with the addition of the cosolvent TFE up to $\approx 12\%$ then remain relatively unchanged for 25 and 50% TFE. However, the longest time constant, τ_3 , increases with the addition of TFE and approaches 70 ps (TFE $\geq 25\%$), which is significantly slower than the time scale of 53 ps obtained with bare Trp in neat TFE.

Multiexponential Decay vs. Stretched Exponential Fit. We have carefully looked into the solvation of Trp and fit the data with the well known exponential types of relaxation pathways (6, 31) and stretched exponential functions over 3 time scales, 0–2 ps, 2–30 ps, and 20–150 ps, as shown in the SI. The data fit is in accord with previous interpretation of a multiexponential fit,

Table 2. Solvation-energy of relaxation, $E_s(t)$, of melittin in different cosolvent concentrations

% TFE	τ_1 , * ps	A_1 , [†] cm ⁻¹	τ_2 , ps	A_2 , cm ⁻¹	τ_3 , ps	A_3 , cm ⁻¹
0	0.397	304 (0.359)	3.78	258 (0.305)	120.7	285 (0.336)
8	0.411	279 (0.235)	3.89	495 (0.417)	91.3	412 (0.347)
12	0.837	344 (0.272)	6.83	402 (0.318)	102.1	519 (0.410)
25	0.713	434 (0.292)	6.84	530 (0.359)	70.8	515 (0.348)
50	0.736	406 (0.267)	6.38	530 (0.349)	60.2	538 (0.384)
100	1.91	256 (0.129)	9.53	617 (0.312)	80.1	1106 (0.558)
0 (Trp) [‡]	0.300	883 (0.558)	1.49	682 (0.431)	13.2	18 (0.011)
100 (Trp) [§]	1.29	474 (0.223)	9.97	696 (0.327)	54.0	957 (0.449)

*Solvation-energy relaxation functions were fit to the relationship, $E_s(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3}$.

[†]Numbers in parentheses are fractional amplitudes.

[‡]Obtained with bare Trp in a phosphate buffer solution (pH = 4).

[§]Obtained with bare Trp in TFE.

and within the experimental errors the fits are significantly better than those of the stretched exponential.

In fitting the solvation during peptide folding, we have considered both the multiexponential and stretched exponential function, which has been introduced to describe protein solvation by Frauenfelder et al. (5). From our protein data, although the signal to noise ratio is slightly less than those of Trp in bulk, we consistently observe a better fit to the discrete exponential function. This is supported by the careful comparison of the component of the bulk type water (τ_1) on the ultrashort time scale and the corresponding one for Trp in the bulk. When we use the exponential fit we obtain good correlation to the bulk type motion (6).

Discussion

TFE Peptide Interactions. The amphiphilic nature of TFE is the key to its structure-inducing properties. The CF₃ group imparts a significant hydrophobicity to the molecule. The electronegativity of the fluorine increases the acidity of the OH group, making TFE an effective proton donor, but a poor proton acceptor. These characteristics allow TFE to reside on the protein surface, displacing the water molecules that compete with intramolecular hydrogen bonds. Many recent studies have suggested that preferential solvation or clustering of fluorinated alcohols is responsible for their exceptional secondary-structure inducing properties, such as the stabilization of the α -helix (19–22). These results indicate TFE and other fluorinated alcohols create a coating around the peptide, expelling water from the peptide surface, and that the density of TFE is ≈ 2 times higher at the surface of the peptide than that of the bulk concentration (20). Fluorinated alcohols such as TFE and hexafluoroisopropanol have also been shown to aggregate appreciably in water-alcohol mixtures (15, 32, 33). These clusters have been reported to form a maximum size at a concentration of $\approx 40\%$ (vol/vol) of TFE (32, 33). At higher concentrations ($>50\%$) the clusters diminish in size because of the increased homogeneity of the solvent. It has been suggested that this propensity for aggregation may act in coordination with their hydrophobic nature to bind with the hydrophobic residues of proteins (10, 34). The hydrophobic aggregates reduce the local polarity surrounding the peptide, resulting in an increase in intramolecular hydrogen bonding (16, 35, 36). There is, however, limited experimental evidence of direct binding of fluorinated alcohols with hydrophobic residues (22, 37).

Tetramer–Monomer Transition. The tetramer–monomer transition is characterized by complex enthalpic and entropic contributions to the overall free energy. Here, we invoke the dominant mechanisms of this transition, which is shown to occur in 2 stages. The first stage of the transition occurs at a TFE concentration of $<12\%$, and is governed by both bulk and local solvent properties. The tight tetramer complex is held together by van

der Waals forces between the close-fitting hydrophobic faces of the monomers and the entropic barrier of binding solvent molecules on the hydrophobic surfaces (38). A pronounced feature of the data are the sudden increase in both the helicity and the local solvent rotation time constant, τ_2 , at a concentration of $\approx 12\%$. The rapid increase in helicity over a range of 5% TFE suggests a 2-state behavior, contrary to the gradual change characteristic of the helix-coil transition (39). Likewise, because τ_2 reflects the molecular rotation and translation dynamics, it should be inversely proportional to the solvent viscosity.

We can estimate τ_2 using bulk viscosity data for water-TFE mixtures, $\tau_2^* = \tau_2(0) \cdot (\eta/\eta_0)$, where $\tau_2(0)$ is the time constant for melittin in neat buffer, η is the viscosity as a function of TFE content (from ref. 24), and η_0 is the viscosity of water. We find that although the bulk solvent viscosity changes by the same magnitude as τ_2 from 0 to 100% TFE (see Fig. 3), the change in viscosity is much more gradual than the abrupt retardation observed in the solvation dynamics. The unexpected suddenness and coincidence of these two different measurements, combined with the property that there is no tertiary structural change for this system, show that both of these events must be triggered by the breakup of the tetramer.

From this observation, 2 important conclusions can be drawn. First, the unbinding of tetramer to monomers is an abrupt transition. Second, when the monomer is separated from the tetramer, Trp is preferentially solvated. In particular, from knowledge of the TFE dependence of the bulk solvent viscosity (25), at the transition concentration we estimate there is at least 40% TFE around Trp, ≈ 4 times the bulk concentration. Previous X-ray and dynamic light scattering experiments (15, 32, 33) and molecular modeling studies (20) suggest that the maximum for solvent clustering in water cosolvent occurs near 30–40% (vol/vol) TFE and that the alcohol concentration is approximately twice that of the bulk concentration; in accord with our solvation measurements. However, we are unaware of another study in which the dynamics associated with solvent clustering near the protein surface have been measured.

Both the helicity and the local solvent rotational dynamics are insensitive to increase in TFE concentration $>12\%$ (see Fig. 3), and thus Trp samples a local environment identical to that of the helical monomers. One may then assume that after this transition at 12% bulk TFE concentration, the monomers are completely free. However, the anisotropy data (T_2) indicate that the effective size of rotating solute does not sharply decrease at 12% TFE, and in fact increases by $\approx 30\%$ (see Fig. 4). Thus, the monomers at this stage are still loosely bound together and partially separated by solvent molecules. It is only with further increase in the TFE concentration ($>30\%$ TFE) that the monomers completely dissociate.

There are thus 2 distinct stages in the tetramer–monomer transition: loosening and complete monomer separation. The

loosening of the tetramer is dominated by local solvent characteristics. The preferential solvation of the peptide surface by TFE eliminates water that competes with intramolecular hydrogen bonds. At a critical concentration the decrease in free energy due to these interactions and the entropic preference for conformational freedom upon loosening is greater than the free energy increase due to the hydrophobic effect. Therefore, TFE penetrates the hydrophobic core, separating the monomers by a layer of solvent, and preferentially solvating the Trp probe. The helicity also experiences a sudden increase because the monomers are no longer sterically frozen in the tight tetramer conformation.

In going from the loosely bound state to the completely dissociated state, the gain in rotational and translational entropy of the monomers (40, 41) must overcome the entropic cost of full solvation and the long-range van der Waals forces (42, 43) holding the monomers together. This occurs at a concentration of TFE >12%. The dissociation of melittin tetramer in 2 distinct stages is a consequence of 2 different entropic barriers: conformational entropy during loosening and rotational/translational entropy during dissociation.

Although the concentration of TFE in the solvation shell may be many times that of the bulk, the network dynamics of this shell evolves in concert with the large amplitude protein motions, and experiences a viscous drag due to its movement relative to the bulk. Therefore, τ_3 (Table 2), which is a measure of the dynamics of the entire solvation shell, does not experience a dramatic change because it is governed by the bulk viscosity rather than the local viscosity. As shown conclusively by Zhong and his group (44) through sequence modification, structural heterogeneity has a relatively small effect on total solvation of proteins.

Conclusions

The solvation of the model peptide melittin provides a unique opportunity for dissecting the role of local and bulk solvent properties in the integrity of protein structure. Melittin exhibits a distinct unfolding of the globular-like tetramer to a loosely bound state in the presence of TFE cosolvent. The location of a solvation probe, tryptophan, in the junction between α -helical monomers within the tetramer allows it to precisely determine the moment of the transition from a tetramer to a loose tetramer. In the loose tetramer state, TFE preferentially solvates the peptide at a concentration many times that of the bulk. It is only with the further addition of TFE that the monomers completely separate. In contrast, the large amplitude peptide motions are associated with the entire solvation shell and bulk viscosity. This study highlights the importance of delineating the multiple time scales for address-

ing the complex sequence of events associated with solvation and protein structural transitions.

Materials and Methods

Sample Preparation and Characterization. Solutions contained 1 mM melittin (purity >95%, Quality Controlled Biochemicals), 10 mM Hepes (Sigma-Aldrich), and 0.5 M NaCl (Sigma-Aldrich), and alcohol content was varied from 0 to 75% (vol/vol) TFE. All samples were used as received without further purification; see the SI for details of sample preparation. An additional sample of 1 mM melittin in neat TFE (purity = 99.8%, Fisher Scientific) was also prepared. The helical content of the melittin solutions was estimated by measuring the ellipticity at 222 nm, at a temperature of 25 °C, and using the program DICHROPLOT (45). Absorption spectra were collected using Cary 500 UV-Vis spectrophotometer and a 0.05-mm path length cuvette. The steady-state fluorescence spectra were measured using a SPEX FluoroMax-3 spectrometer with excitation at 290 nm. Trp in a buffer solution exhibited a fluorescence maximum at 353 nm, which shifted to 339 nm in neat TFE. All measurements were made at room temperature unless otherwise specified.

Time-Resolved Fluorescence Measurement. To determine the fluorescence lifetime of Trp, the time correlated single photon counting (TCSPC) measurements were carried out using pulses (<100 fs) from a Ti-sapphire oscillator (Mai Tai HP, Spectra Physics). The oscillator output wavelength was tuned to 870 nm and frequency-tripled to 290 nm using a time-plate tripler (TP-2000B, MiniOptic Technology) for excitation of Trp. The excitation pulse energy was attenuated to ~ 10 pJ. The fluorescence was detected at the magic angle (54.7°) with respect to the vertically polarized excitation beam and focused into a micro channel plate photomultiplier tube (R3809U, Hamamatsu) attached to a double monochromator. Multiexponential decays convoluted with instrumental response functions (measured using a standard scattering solution, typically 30 ps) were analyzed using the FluoFit software package (PicoQuant).

Femtosecond-resolved transients were obtained using the fluorescence up-conversion technique. We used the output of an amplified Ti-sapphire laser system (Hurricane X, Spectra Physics), which produces ~ 110 -fs pulses centered at 804 nm (fundamental) with a 1-kHz repetition rate and a 0.8 mJ energy. Further experimental details are outlined in the SI. The temporal response of the instrument was 350 ± 20 fs. The fluorescence transients were fit to theoretical functions, using the Scientist nonlinear least-squares fitting program (Micromath), for the convolution of the Gaussian instrument response function with a sum of exponentials. These transients were used to reconstruct the solvation energy change as described by Lu et al. (46).

Time-dependent fluorescence anisotropy, $r(t) = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, was determined from transients recorded at a fluorescence wavelength of 350 nm. The pump beam with parallel ($I_{\parallel}$) or perpendicular ($I_{\perp}$) polarization were set with respect to the acceptance angle of the up-conversion crystal.

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