Editorial

A new look for the RLBL Newsletter

By Thomas Troxler

This is the 25th issue of the RLBL newsletter. We decided to celebrate this event with a new layout of our publication, designed to enhance the presentation and ease access to new information. One such improvement is the small content box in the left bottom corner of this page, which summarizes the current issue at a glance. Both the editorial and our feature article begin on the first page side by side and continue as indicated. They are followed by the research articles. After the last research article we maintain a short description of all experimental capabilities of the RLBL, as well as a list of recent publications in order to keep the community up to date about current activities at this NIH resource center. As always, the last page contains an application form to use the RLBL.

Feature Article

Observing helix formation with IR Laser Spectroscopy


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The helix-coil transition kinetics of an α-helical peptide were investigated by time-resolved infrared spectroscopy coupled with laser-induced temperature jump (T-jump) initiation method. Specific isotope labeling of the amide carbonyl groups with 13C at selected residues was employed to obtain site-specific information.

Introduction

The protein folding problem is considered to be one of the fundamental questions in structural biology. Folding research is today a very active area, and both experimental and theoretical techniques for probing the protein folding process at the molecular level are becoming more and more refined. Given the primary amino acid sequence, it is the goal of all these studies to provide a basis for understanding and predicting protein folding pathways, the stable and biologically active structures, and thermally and kinetically accessible conformation substates. Understanding how proteins fold helps to interpret quantitatively structure-function relationships, folding related diseases, and is apparently important for protein engineering (1).
New executive committee member

We would like to take the opportunity and to introduce in this issue a new member of our executive committee, Professor Feng Gai. Professor Gai joined the faculty of the Chemistry Department of the University of Pennsylvania in 1999 as assistant professor. He has previously served as postdoctoral fellow at Harvard University and the Los Alamos National Laboratory. The focus of his research is the study of how proteins fold from a random or quasi-random coil to their biologically functional conformations. Some of his experimental approaches include laser-induced temperature jumps, rapid mixing, infrared, fluorescence, and single-molecule spectroscopy. Time resolved methods allow him and his research group to study the early events in protein folding, which are difficult to access by other means. An example of such a study is given in the feature article of this issue.

Another member of our executive committee, Prof. Michael J. Therien contributed a second research article to this issue. He and his students/postdocs have recently set up an ultrafast transient absorption spectrometer, and the capabilities of this instrument are clearly portrayed in the article starting on page 8.

Last, but not least, we also include an article about an exciting new application in the field of single molecule spectroscopy. This article contributed by Minyang Lee et al. contains the first experimental test of the distribution of fluorescence lifetimes that are expected from fluorescence resonant energy transfer, allowing for an in depth analysis of theoretical models.

Updated web site

We recently also redesigned our whole web site at http://rlbl.chem.upenn.edu, which we encourage you to visit. An extended description of the resources available to outside users is included, as is information about new instrumentation as it becomes available for collaborative or service projects. Please contact us if you are interested in any of the instruments and approaches to discuss possible collaborative and service projects.♦

It is well known that many proteins can spontaneously fold or unfold in response to renaturing or denaturing conditions (2), suggesting that the primary amino acid sequence encodes the final native structure and information on how to get to this conformation. Although the molecular details of protein folding have been subjected to intensive studies, a quantitative knowledge of the relation between the primary amino acid sequence and the final folded state, as well as the pathways connecting final states, are still not attainable. Part of the difficulty is the fact that there is no universal probe available that provides conformational snapshots in the course of folding with sufficient time and structural resolution. Nevertheless, measurements on folding kinetics and dynamics over a very broad time range are essential to arrive at a detailed understanding of the folding problem. Clearly, techniques that have the ability to initiate folding, refolding or unfolding with sufficient time resolution and structural sensitivity are needed.

The energy landscapes of short helical peptides are expected to be much simpler than of proteins and therefore have attracted the attention of both theoretical studies and computer simulations. MD simulations using an all-atom peptide model in explicit solvent have shown that the helix formation kinetics of an Ala pentapeptide can be described quantitatively by a diffusive search within the coil state with barrierless transitions in the helical state. Such a diffusive model should be testable experimentally by T-jump induced conformation changes. Furthermore observed relaxation in T-jump experiments should be sensitive to the width of the T-jump since conformation diffusion should depend on both the effective diffusion constant and the initial conformation distribution, determined by the initial and final temperatures.

Materials and Methods

A standard Fmoc-protocol employing Pal resin was used for peptide synthesis. All samples were purified to homogeneity and characterized by electrospray-ionization mass spectroscopy. For samples used in the infrared experiments, the residual trifluoroacetic acid (TFA) from peptide synthesis, which overlaps with

continued on the next page
the amide I band at 1672 cm⁻¹ in the IR spectrum, was removed by lyophilization against DCI. For both equilibrium and time-resolved IR experiments, the peptide was dissolved directly in D₂O with a final concentration of 2-4 mg/ml.

The sequence of the four α-helical peptides studied are Ac-YGSPEA₃K₄r-CONH₂ (nonlabeled peptide), Ac-YGSPEA₃K₄AA₄K₄r-CONH₂ (ML peptide), Ac-YGSPEA₄KA₄K₄r-CONH₂ (NL peptide), and Ac-YGSPEA₄KA₄K₄AA₄r-CONH₂ (CL peptide), where underlined residues are ¹³C-labeled and r represents D-Arg.

To access and characterize the early folding events requires a fast initiation method and a probe that has structural specificity. A T-jump coupled time-resolved infrared apparatus was developed to measure relaxation kinetics at discrete frequencies with nanosecond time resolution and high signal to noise (S/N) ratio. The current setup is tunable from 1550 cm⁻¹ to 1800 cm⁻¹, which covers the entire amide I' band region of proteins. The schematic of this T-jump infrared spectrometer is shown in Figure 1.

Briefly, a 3 ns, 10 mJ, 1.9 μm, and 10 Hz T-jump pulse was generated by Raman shifting the Nd:YAG fundamental at 1064 nm in a mixture of H₂ and Ar gas pressurized at 750 psi. The resulting 1.9 μm pulse has a characteristic noise level of 3% or less. A low noise pump source is critical to obtain repeatable and clean results in any T-jump experiment because of the intrinsic dependence of the observed signal on temperature, the latter depending on the pump energy. A T-jump of 10-15 °C could be obtained routinely in an approximately 80 nL laser interaction volume (1 mm spot size X 0.1 mm path length). The magnitude of the T-jump is calibrated with the D₂O absorbance change. A CW lead salts infrared diode laser from Laser Analytics serves as a fixed wavelength probe, which is tunable from 1550 to 1800 cm⁻¹. Other frequencies may also be covered with additional laser modules. Transient absorbance changes of the probe induced by the T-jump pulses were detected by a 50 MHz MCT detector from Kolmar Technologies, and digitized by an oscilloscope. In all experiments a sample cell with dual compartments and a 100 μm path length was used to allow for the separate but parallel measurements of the sample and reference buffer under identical conditions. The IR absorption spectrum of the buffer (mostly D₂O) is temperature dependent near the amide I' band, thus quantitative subtraction of the reference spectrum at each temperature is essential.

Results

Static FTIR spectra of the labeled and nonlabeled peptides in the amide I' region (Figure 2) were collected roughly every 7 °C, from ~3 °C to ~76 °C. All these spectra were fully reversible upon cooling, and no aggregation was detected. At low temperature, the nonlabeled peptides exhibits an amide I' band at ~1636 cm⁻¹, typical for solvated helices. Its labeled derivatives show two major bands at ~ 1600 cm⁻¹ and 1640 cm⁻¹, respectively. We assign the 1600 cm⁻¹ band to the corresponding amide I' absorbance of the ¹³C-labeled residues. FTIR difference spectra (Figure 2) show directly that the amide I'-band loses intensity as temperature increases, and the concurrent formation of new spectral features at higher wavenumbers.

The intensity of the 1600 cm⁻¹ component of the CL peptide is much smaller than those of the NL and ML peptides, which was recently attributed to the occurrence of end fraying at the C terminus (3,4). Calculation based on classical helix-coil transition theory (5,6) and the zipper model of Eaton et al. (7) also show also that the helical content at the C-
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Terminus is reduced in comparison with the N-terminus or the middle of the peptide chain. Theoretical models and experimental results have suggested that short helical peptides contain helices of various lengths (7,8), and an exact two-state description of helix-coil equilibrium is inadequate. The thermal unfolding curves measured by IR of the labeled and unlabeled peptides indeed showed different behaviors as seen in Figures 3, clearly indicating deviation from a two-state transition. These results suggest that a helix containing the least number of residues in the bracket, Ac-YGSPE[A3K][A4]KA4R-CONH2, is probably the dominating helical species. This figure also shows that specifically isotope labeling residues at selected positions provides an effective means to probe site-specific conformational changes.

The relaxation kinetics of the three labeled peptides following a T-jump of ca. 10 °C, from 5 °C to 15 °C, probed at 1600 cm⁻¹, reveal small but noticeable differences between that of the CL peptide and that of NL or ML peptide. Figure 3 plots the difference in optical densities as a function of time after the 10 ns T-jump within a window of 1.4 µs. On the other hand, the relaxation kinetics of the NL and ML peptides remain identical within experimental errors. All data were nicely modeled by a stretched exponential function (9) and an instantaneous component that is not resolved due to the limited response time (7-10 ns) of the IR detection system. Specifically, the function used to fit the data is \( \Delta \text{OD}(t) = A[1-B \exp(-t/\beta)] \), where \( A \) is the full amplitude and \( (1-B) \) is the percentage of the instantaneous component. \( 0 < \beta < 1 \) is a measure of the extent of deviations from single exponential kinetics. All parameters obtained from least square fitting are listed in Table 1. It is evident from these results that the relaxation kinetics of the C-terminus, following a T-jump, are faster and more stretched than that of the middle or N-terminus of the peptide.

Another significant difference is the larger percentage of the instantaneous component in the case of the CL peptide, but the nature of this component remains elusive, as two mechanism may contribute to this fast phase, e.g. an instantaneous spectral shift of a conformational change of a subnanosecond time scale. In a similar experiment employing fluorescent probes, Eaton et al. (7) recently attributed the addition/removal of a hydrogen bond to an existing helix to be responsible for a very fast initial relaxation. It is likely that the fast component in our case is at least partly due to similar processes. But the overall faster relaxation of the helical content near the C-terminus indicates that the unwinding of the helix most likely occurs through the initial breaking of hydrogen bonds at the C-terminus, also suggested by other studies (1,10).

More surprising was our finding that the T-jump induced relaxation kinetics are dependent on the T-jump widths, i.e. the relaxation is sensitive to both initial and final temperature. Although T-jump width dependent relaxation kinetics have been observed for proteins (11,12), it was not anticipated that peptides containing only secondary structural elements, such as the \( \alpha \)-helix, can also exhibit such kind of behavior. As an example of our observations, Figure 4 shows the measured relaxation kinetics of the ML peptide, again probed at 1600 cm⁻¹, under two different T-jump conditions. In this case, two different initial

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**Figure 2**: Temperature dependent equilibrium (top traces, 3ºC – 76ºC) and difference (bottom, 31ºC – 76ºC) FTIR spectra of the nonlabeled and one labeled (ML) peptide. The bands at ~ 1600 cm⁻¹ and ~1636 cm⁻¹ are assigned to the amide I' absorbance of the 13C-labeled and nonlabeled residues.
temperatures of ~ 0.5 °C and ~ 10 °C were employed, but the final temperature was ~14.5 °C in both cases. From Figure 4 it is apparent that the T-jump induced relaxation depends on the initial temperature, with a relation that larger T-jumps give rise to faster overall relaxation rates. Again, these relaxation kinetics can be modeled by stretched exponential with an instantaneous component. Fitting results from four different initial temperatures are summarized in the lower part of Table 1. Note that the time constant of the stretched exponential seems to exhibit a linear dependence on the T-jump amplitude ΔT, at least within the range of temperature changes probed in this study. Extrapolating ΔT to zero results in a relaxation time constant of ~ 390 ns for the final temperature of ~14.5 °C. This time should correspond to the exchange time obtained from 13C NMR transverse relaxation experiments (13) that have been done at the same final temperature.

Discussion

Stretched exponential kinetics has been observed previously in many condensed matter systems as well as in proteins. Explanations for this behavior include diffusion processes with correlated traps, the existence of a broad distribution of relaxation lifetimes due to inhomogeneity, or the modulation of rates of an underlying exponential process. In the case of proteins, Dobson et al. (4) have reported that the refolding kinetics of equine lysozyme follow a stretched exponential law, as well as the refolding kinetics of cold denatured phosphoglycerate kinase, studied by Gruebele et al. (12) However, it was less anticipated that short helical peptides as used in the current study could follow such a complex relaxation. These deviations from single exponential relaxation kinetics clearly indicates that a simple and rigorous two-state model cannot account for the nature of the helix-coil transition even in these small chain helical peptides. Models including intermediate states or multiple folding pathways have to be used, also seen in several simulation results of biological or non-biological helices. For example, in a recent all-atom computer simulation Pande et al. (15) reported the folding via an on-pathway intermediate in the case of a non-biological helix of poly-phenylacetylene. Also, Wu et al. (10) found that frequent transitions between a 3_10-helix and an α-helix occur in their molecular dynamics simulation. Nonetheless, all these interpretations can hardly explain the fact that the relaxation kinetics also depends on the initial temperature as we observed in our model study.

Using the concept of protein folding energy landscape, an alternative explanation can be derived for our observations. In this model folding is described as a
diffusion-like process on a highly dimensional energy surface biased toward the native state, with the mean folding time determined by both the overall shape and ruggedness of the energy landscape. The folding of a short peptide would be seen as a downhill diffusion in conformation space without significant free energy barriers to the native state. Support for this model also comes from all-atom MD simulations with an explicit solvent of the simplest peptide that can form 1.5 turns of an α-helix: Ac-Ala5-NHMe (A5 peptide) done by Hummer et al. (1). In this case nucleation occurs very fast on a time scale of 100 ps. The coil-to-helix transition of A5 peptide can be described quantitatively by a diffusive search within the coil state with barrierless transitions into helical conformation (16), whereas the helix-to-coil transition occurs predominantly through the breaking of hydrogen bonds at the helix end, strongly favoring the C-terminus.

Two conclusions from this model can be tested by T-jump coupled folding experiments. First, a conformation search with coil states to helix states would exhibit nonexponential kinetics. Coils close to the helical state would relax faster than coils distant from the helical state in conformation space. Second, a diffusion search model would suggest that the helix formation depend on the initial distribution of the coil conformation. This would lead to T-jump width dependent relaxation kinetics in the T-jump experiments, as observed in this study.

To explain the fact that the mean relaxation time gets longer when ΔT approaches zero (Figure 4b) in our experiments, a pseudo two-state model is applied. The free energy profile of a helical peptide can be depicted as two broad basins with a barrier separating the coil ensemble from the helical ensemble (17,18). The two ensembles are in thermal equilibrium at any given temperature. A T-jump induced relaxation causes the coil population to increase, but the observed signal contains contribution from both folding and unfolding. This is the case because the T-jump experiment is essentially a relaxation method. Since the final temperature of ~14.5 °C is smaller than the thermal melting temperature of ~16 °C, the overall folding rate is comparable to the overall unfolding rate. Therefore we attribute the observed nonexponentiality and the T-jump width dependence of the relaxation kinetics mainly to the folding of the coil conformation. A sudden change in temperature will project both the coil and helix populations to a new free energy surface.
different from the one before the T-jump. However, the initial population distribution is unchanged (Figure 5). This causes the coil states on average to be closer to the helical states when the initial temperature is the coil states are relatively distant from the helical state, it takes longer for them to diffuse to the helical region.

In summary, the T-jump induced helix-coil transition kinetics of an Ala based helical peptide, obtained by probing the amide I' band of the peptide backbone with IR spectroscopy, revealed nonexponential behavior and dependence on both initial and final temperatures. Our results provide strong evidence supporting the picture that the nucleation process is fast, on a nanosecond or even subnanosecond time scale, and the helix formation process can be described by a diffusion search within the coil states. Further studies will be carried out to address questions as to how peptide stability and length influence its folding dynamics, and what the nature of the fast component is.

Acknowledgment
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Literature
9. In an early publication (JACS 123, 12111 (2001)), a multiexponential function instead of a stretched exponential function was used to fit the data. Also in an early publication (JACS 123, 9235 (2001), nonexponential relaxation was not observed due to slower time resolution.
SYNTHESIS, EXCITED-STATE DYNAMICS, AND REACTIVITY OF A DIRECTLY-LINKED PYROMELITIMIDE-(PORPHINATO) ZINC(II) COMPLEX

By Namoi P. Redmore, Igor V. Rubtsov, and Michael J. Therien

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N-[5-(10,20-Diphenylporphinato)zinc(II)-N'-(octyl) pyromellitic diimide (PZn-PI) has been synthesized and the interrogation of its photo-induced charge separation and thermal recombination electron-transfer dynamics via ultrafast pump-probe transient absorption spectroscopic methods is reported. An unusual reactivity of PZn-PI with respect to many directly linked donor-acceptor compounds was found.

Introduction

Light absorption, exciton delocalization, energy transfer (E-T), and electron transfer (ET) define the key events of biological energy transduction. For example, the biological light harvesting complexes (LHCs), along with the electron transfer apparatus of photosystems I and II, are replete pigment-pigment and donor-acceptor interactions that feature substantial electronic coupling. During photosynthesis, fast multistep ET reactions transform the absorbed energy into chemical potential energy with very high efficiency. Porphyrin-quinone (P-Q) compounds, in which the quinone is directly linked to the porphyrin macrocycle, have served as biomimetic benchmark systems for charge separation (CS) and thermal charge recombination (CR) reactions that involve strong donor-acceptor (D-A) electronic coupling (1-10).

A closely related D-A system in which a pyromellitimide (PI) acceptor is directly fused to the meso-carbon of a (porphinato)zinc(II) (PZn) chromophore is reported here. We contrast its ET dynamics with those established previously. This new D-A unit will serve as a key building unit for the future synthesis of more complex ET assemblies.

Experimental Section

Materials. A detailed description of the synthesis of PZn-PI is given in Ref. 11. All manipulations were carried out under nitrogen, and standard Schlenk techniques were employed to manipulate air-sensitive solutions. Chromatographic purification of all newly synthesized compounds was accomplished on the bench top (see Figure 1 for a reaction scheme).

Pump-Probe transient absorption measurements.

Transient absorption spectra were obtained using standard pump-probe methodology. Ultrafast optical pulses, centered at 775 nm, were generated using a Ti:sapphire laser (Clark-MXR, CPA-2001). Optical parametric amplifiers (near-IR and visible OPA) generate excitation pulses tunable in wavelength from the UV through the near-IR region. A white light continuum, generated by focusing into a sapphire window, served as a probe beam. After passing through the sample, the probe beam was focused onto the entrance slit of a computer-controlled image spectrometer (SpectraPro-150, Acton Research Corp.). Typical noise level in these transient absorption experiments correspond to ~0.2 mOD/s of signal accumulation. The time resolution varies between 140 fs and 200 fs depending on the probe wavelength. All experiments were carried out at room temperature (23±1 °C).

Figure 1: Synthetic route and structure of PZn-PI.
Results and Discussion

Early work established the utility of the PI acceptor in porphyrin-based donor-spacer-acceptor (D-Sp-A) assemblies (12,13). This species is a strong electron acceptor \( (E_{1/2}(PI-/PI) = -0.8 \text{ V vs SCE}) \) (14). Additionally it features a radical anion absorbance with significant oscillator strength. The PI moiety thus provides an excellent spectral signature for monitoring the growth and decay of ET intermediates.

Contrary to optical spectra of P-Q systems, the electronic spectra of PZn-PI corresponds well to 10,20-diphenyl-(porphinato)zinc(II) compounds with a strong Soret band around 410 nm and a weaker Q-band centered at 535 nm. This shows that the perturbation to the porphyrin \( S_0, S_1 \) and \( S_2 \) electronic states upon appendage of the PI acceptor to the (porphinato)zinc(II) chromophore is not severe.

Figure 2: (A) Transient absorbance spectra obtained following excitation of PZn-PI in methylene chloride at 549 nm at the labeled time delays. (B) Exemplary wavelength-dependent transient decay curves.

The photoinduced and thermal charge recombination ET dynamics of PZn-PI in \( \text{CH}_2\text{Cl}_2 \) were investigated by pump-probe transient absorption spectroscopy (Figure 2). PZn-PI was excited by a 120 fs pulse (\( \lambda = 549 \) nm), resulting in the formation of a porphyrin-based doubly degenerate singlet excited state \( \{1\text{(PZn)}\}^*\text{-PI}, \) see Figure 3B. The subsequent charge separation reaction can be fit as a monoexponential process with a time constant of \( CS \) of 770 fs. The transient absorption spectrum corresponding to a time delay of 2.0 ps (Figure 2) displays the classic spectral signatures for both the PI anion radical(13) and the PZn cation radical(15); note that the characteristic respective absorbances (710 and 470 nm) for these two species are clearly evident. This charge-separated state undergoes a rapid charge recombination reaction \( (CR = 5.2 \text{ ps}) \), producing a vibrationally hot ground state. The hot ground state cools with a time constant of 17 ps in methylene chloride, similar to that observed for strongly coupled PQ benchmarks.(5) It is important to note that similar transient dynamics are observed for PZn-PI irrespective of the excitation wavelength within the Q-state absorption manifold.

Figure 3: Summary of the proposed electron-transfer dynamics for (A) PMg-Q and (B) PZn-PI.

It is instructive to compare the ET dynamics manifest by PZn-PI with that displayed by a related P-Q compound, (5-quinonyl-10,15,20-triphenylporphinato)magnesium(II) (PMg-Q). Ultrafast time-resolved spectroscopy demonstrates extensive mixing between the quinone moiety and the (porphinato)metal \( x_\nu \)-polarized Q state.(1) Excitation within the Qx absorption band \( (hv, \) Figure 3A) produces a charge-transfer (CT) excited state, \( [P+\text{Mg-Q}^-]^* \), which decays via rapid internal conversion to a vibrationally relaxed CT state \( (P+\text{Mg-Q}^-, \) Figure 3A) within a subpicosecond time domain (1). In contrast, electronic
excitation within the Qy absorption envelope (hv, Figure 3A) affords a porphyrin-localized excited state [1(PMg)*-Q], which decays via a solvent-independent ET process to produce the charge-separated state (P+Mg-Q-) on a time scale (CS = 350 fs) likely rate-limited by electronic population equilibration between the (porphinato)metal Qx and Qy levels.(1) Notably, the ET dynamics evinced for PZn-PI are pump wavelength independent within the Q-band spectral region (Figure 3B), indicating that no direct CT states are accessed over these excitation energies. These data suggest that a combination of diminished D-A electronic coupling and increased activation free energy in PZn-PI may be responsible for the observed differences in the excited-state dynamics relative to those elucidated for PMg-Q.

Conclusions

In summary, we have prepared a meso-pyromellitimide-substituted (porphinato)zinc(II) complex which undergoes ultrafast, photoinduced charge separation and thermal charge recombination ET reactions. This ultrafast ET dynamics observed for PZn-PI in methylene chloride differ markedly from those delineated previously for closely related 5-quinonyl elaborated porphyrin compounds, such as PMg-Q (1). Notably, the PZn-PI complex can be further functionalized via halogenation and subsequent metal-catalyzed cross-coupling at the porphyrin meso-position anti to the PI moiety: we thus expect that this species will be a useful synthon in the preparation of more elaborate multichromophoric donor-acceptor arrays.

Acknowledgment

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Literature

Research Article:

Fluorescence Lifetime Distribution of Single Molecules Undergoing Förster Energy Transfer

By Minyung Lee, Jianyong Tang, and Robin M. Hochstrasser

Chemistry Department, Ewha Womans Univ., South Korea, and Chemistry Department, University of Pennsylvania

Comment:
The RLBL is involved with a number of single molecule energy transfer experiments. The following describes one of our efforts to understand basic properties of FRET in relation to single molecule experiments.

Introduction

When a donor molecule surrounded by energy acceptors is electronically excited, its fluorescence decay exhibits a single exponential, owing to the Förster energy transfer. On the other hand, an ensemble excitation of the donor shows a nonexponential decay function of \( I(t) \), resulting from a distribution of the energy transfer rate processes. The bulk decay function can be written as an average of the decay rates:

\[
I(t) = \left\langle \exp\left(-kt\right)\right\rangle = \int_0^\infty P(k) \exp\left(-kt\right) dk \quad (1)
\]

where \( P(k) \) is the rate constant density distribution, so that the probability of the energy transfer rate constant being close to \( k \) is \( P(k)dk \). The probability distribution function can be converted to a fluorescence lifetime distribution or a distance distribution. If there is no a priori knowledge about the distribution, one must determine it from \( I(t) \). This is tricky since even small experimental errors in the decay curve produce quite different distribution functions [1]. There exist several algorithms such as the maximum entropy method (MEM) [2], CONTIN [3], and others that are used to find \( P(k) \) numerically[4-6]. They have not been tested with experimental distribution data because the bulk decay function and \( P(k) \) have never been measured together in the same system. The probability distribution of the energy or electron transfer rates of single donor-acceptor pairs have been studied by numerous investigators, using single-molecule confocal fluorescence microscopy, but such measurements determine only pair distribution functions [7-14].

In this work we obtained the decay rate constant distribution \( P(k) \) through single molecule fluorescence lifetime measurements. Understanding the correct distribution form of acceptors is very important in the field of Förster resonance energy transfer (FRET) in restricted geometries as well as in homogeneous environments, sensor applications, and cell receptor binding of ligands.

Experimental

Lifetimes of single tetramethylrhodamine ethyl ester (TMR) molecules were measured with a home-built scanning confocal microscope, having a time-correlated single photon counting (TCSPC) capability. Details of the single-molecule fluorescence detection system at RLBL were described elsewhere [7]. To avoid any complexity arising from donor and acceptor translational diffusion, the donor, TMR and acceptor azulene, were immobilized. The donor is one of the most photostable visible dyes [15]. The acceptor molecule has its absorption band in the visible (430-730 nm) and its contribution to background fluorescence is extremely low because of its ultrafast internal conversion (<2 ps). At RLBL we have discovered that having negligible emission from the acceptors is a great advantage for the single-molecule energy Förster transfer experiment.

Results and Discussion

The fluorescence lifetime decays of individual single molecules were single exponentials. The distribution was symmetric with a mean value of 3.15 ns and a standard deviation of 300 ps, as shown in Figure 1(a). Figure 1(b) shows the comparison of the single molecule data with bulk, which are in close agreement.

In the presence of azulene molecules, the TMR lifetime \( \tau_D \) is shortened due to resonance energy transfer. When there are \( N_A \) acceptors, \( k_T \) is given by...
\[ k_T = \sum_i \frac{N_i}{\tau_i} \frac{3}{2} \kappa_i^2 \left( \frac{R_{\text{eff}}}{R_i} \right)^6 \]  \hspace{1cm} (2)

where \( k_i \) is the energy transfer rate constant for the donor and the \( i \)th acceptor, which depends on the distance \( R_i \) and the orientation factor from the dipole-dipole interaction \( \kappa_i^2 \). The Förster radius for the TMR-azulene pair is 30 Å. The bulk fluorescence is not a single exponential, but becomes non-exponential, owing to the distribution of donor decays. For \( N \) donors, the normalized bulk decay curve is the discrete form of Eq. (1)

\[ I(t) = \sum_{j=1}^{N} P(k_j) \exp(-k_j t) \]  \hspace{1cm} (3)

so that, if the rate constant histogram of single molecules can be obtained experimentally, the bulk decay curve can be recovered. Förster, in his initial development of the resonance energy transfer theory, derived an analytical form for the nonexponential decay profile by a statistical averaging method [15].

When donor and acceptor translational diffusion are negligible, the Förster equation is:

\[ I(t) = \exp\left[-\left(at + bt^{1/2}\right)\right] \]  \hspace{1cm} (4)

with \( a = 1/\tau_D \) and \( b = g \frac{4}{3} \pi^{1/2} n_A R_0^3 / \tau_D^{1/2} \)

where \( n_A \) is the acceptor number density, and \( g = (3\kappa^2>/2)^{1/2} \). In the present case, we have a fixed donor and a random distribution of fixed acceptors, a condition that leads to a static averaging limit \( (\kappa^2) = 0.476 \) [16]. We found a \( b \) value of 0.61 ns\(^{-1/2}\).

Figure 1: (a) Fluorescence lifetime distribution of single molecule TMRs in PMMA. The solid line is a Gaussian fit to the data. (b) Fluorescence decay profile of bulk TMR in PMMA. The solid line is the single-exponential fit to the data and the broken line is the peak-normalized sum of the single molecule lifetime data shown in Figure 1(a).

Figure 2: (a) Fluorescence decay profile of bulk TMR in PMMA with azulene concentration of 1.4x10\(^{-2}\) M. The solid line is the double exponential fit to the data. The instrument response function is also shown. (b) Typical fluorescence decay profiles of single molecule TMRs in the presence of azulene in PMMA.
The average nearest-neighbor distance in the sample is given by \(0.554/n_A^{1/3}\) in which \(n_A\) is the number density [17]. The calculated mean value of 27 Å is equal to the Förster radius of the TMR-azulene pair in this case. Because the bulk data fit both a double exponential form and the Förster decay law, we have another example of the lack of uniqueness in the creation of a distribution from a time evolution.

We carried out the lifetime measurement of the TMR single molecules in the presence of azulene acceptors and some of the typical decay data are shown in Figure 2 (b). A total of 244 single TMRs were chosen by objective procedures and their fluorescence decays were obtained with the RLBL TCSPC instrument. Figure 3(a) shows the distribution of the observed lifetimes which peaks around 2.2 ns, but is highly asymmetric to the short decay time side. It is interesting that the maximum fluorescence lifetime is similar to the longer component of the bulk data fitted to two exponentials. The sum of all the single molecule decay functions is compared with the bulk data in Figure 3(b) and the single molecule data deviates from the bulk decay curve at the earliest times. This is consistent with some of short components being omitted from the lifetime distribution. Such omissions are expected because the molecules that are brightest are unavoidably selected preferentially.

If the bulk decay follows the Förster equation, the probability density function should be the inverse Laplace transform of \(I(t)\) of Eq. (1):
To compare the experiment with Eq. (5), the lifetime distribution was converted to a histogram of the decay rate constant k and plotted in Figure 4 (a). The fit is very good in the slow rate constant region, but deviates strongly starting from the rate constant of $0.8 \times 10^9$ s$^{-1}$. Nonetheless, the b value obtained from the fit is close to that obtained from the bulk data. Of course, $P(k)$ of Eq. (5) is different from the real distribution because the Förster decay law is itself an approximate solution. For example, it does not consider the excluded volume effect. The experimentally observed distribution deviates somewhat from the theoretical PDF for any reasonable choice of b. This deviation is significant but will require many more measurements before a quantitative analysis will be possible. However, the histogram of the lifetime distribution of single TMR molecules in the presence of azulene is compared with that obtained from the Förster equation with different b values. The experimentally observed distribution deviates monotonously in the short lifetime region. On the other hand, the theoretical curve shows a maximum above 2.2 ns and minimum around 1.0 ns. Interestingly, the probability density of single molecules increases again, as the distance between the donor-acceptor molecules gets smaller.

In our system where the average nearest neighbor distance of donor and acceptor is equal to the Förster radius of the donor-acceptor pair, the TMR lifetime distribution is very broad, ranging from 200 ps to 3.2 ns, and highly asymmetric and it deviates from that deduced from the Förster equation. Although the bulk data can be fit well by a double exponential function, the fluorescence lifetime distribution of single molecules confirms that such an interpretation has no physical basis.

These experiments really require that thousands of molecules be examined in order to obtain reliable distributions. The multiplexing of single molecule lifetime measurements is one of the immediate goals of the RLBL.

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References

CURRENT TECHNOLOGICAL DEVELOPMENT AND RESEARCH AT THE RLBL

The main subjects under investigation at RLBL are shown below. If your research may be interfaced with any of these approaches we urge you to contact us. A full description of each of these topics is also available at our Web site http://rlbl.chem.upenn.edu.

- **Dynamics of photoactivatable proteins and other biological structures**: Methods are being developed to examine the responses of biological systems to light by pump/probe and nonlinear spectroscopic methods encompassing spectral regimes from the UV to the far IR and covering femtosecond to second timescales. Techniques include: single and multiple wavelength transient spectroscopy (UV/Vis, vibrational IR, Terahertz), photon echoes, two photon absorption and time-correlated single photon counting.

- **Methodologies to investigate protein folding and macromolecular conformational dynamics**: Detection and characterization of intermediate states in conformational dynamics and unfolding is another developing technology at RLBL. Laser-based temperature-jump instruments are available for these investigations.

- **Investigations of single molecular assemblies using confocal and atomic force microscopes**: It is now possible to examine the properties of single molecules using fluorescence in association with confocal microscopy. The RLBL is coupling single molecule detection methods with mature time correlated photon counting technology, polarization scanning and pulsed laser experiments.

- **Energy transfer and fluorescence monitoring of biological dynamics**: Monitoring fluorescence lifetimes and anisotropies reveals details of protein dynamics. Techniques are being developed at the RLBL to monitor these properties of fluorescing species on the femtosecond to nanosecond timescale.

- **Development of time resolved far-IR (terahertz) probes for protein dynamical changes**: New powerful sources of THz and far-IR radiation are developed and used as laboratory THz source.

- **Two-dimensional infrared spectroscopy and infrared analogues of NMR**: Heterodyned photon echo spectroscopy and spectrally resolved three pulse IR photon echoes are employed to investigate the amide I region and other transitions of peptides and small proteins.

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Logistics: Equipment to be supplied by applicant, needed from RLBL, and anticipated time.

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