LOCAL NEWS:
Changes in staffing have occurred at the RLBL since Newsletter 16 was published. Scott Williams, our Laser Research Specialist, has taken a faculty position at the Rochester Institute of Technology in Rochester, NY. We wish Scott well in his future endeavors. His replacement is Dr. Luis Jahn, who is an expert in the use of ultrafast laser techniques. He graduated Ph.D. from the University of Rochester in the research group of R.J. Dwayne Miller. Later he was a staff member at the Center for Photoinduced Charge Transfer at the University of Rochester.

New laser systems are coming on line at the RLBL which utilize broadband emitting solid state materials, most notably Ti:sapphire. We are applying these systems for the creation of ultrafast light pulses from the UV to the mid-IR by using nonlinear generation and amplification techniques in a variety of crystals (KTP, LBO, BBO, AgGaS$_2$, etc.). This allows us to pump and probe virtually anywhere in the spectrum, giving us maximum flexibility to observe important photophysical processes in biologically relevant systems. In addition, we are developing a mJ pulse energy ultrafast system for high peak energy (terawatt) spectroscopic investigations.

The RLBL is also getting a major “facelift”. With the help of the University of Pennsylvania and the Chemistry Department we are in the process of renovating existing office and lobby space in the Facility as well as creating a reading room and computer area for our users. These improvements will certainly brighten up the facility and increase the productivity of ongoing research.

In mentioning the computer area, I must also add that we have recently obtained a high-powered personal computer with Internet capabilities. There are very important benefits that result from the utilization of this computer: 1) analysis of data will be increased dramatically at the Facility, especially with increasingly larger data sets from the new high rep rate lasers and 2) a “home page” on the World Wide Web will be set up in order to facilitate the efficient exchange of new and exciting information surrounding the Facility.

I am currently in the process of creating this home page and it will be up and running very soon. The address for the home page is http://rlbl.chem.upenn.edu. If you have the appropriate software (i.e. Mosaic, Netscape, etc.) you will be able to view it. I would also appreciate that any comments or suggestions about the home page be sent to my e-mail address, which is located at the bottom of this page.

NEWSLETTER SUBJECTS:
We have been continuing our core research project which investigates the response of the protein RNaseA to an ultrafast temperature rise in aqueous solution and the concomitant unfolding of this protein monitored by IR spectroscopy. This project forms the subject of the highlight article in this issue of the Newsletter. In addition we have an article, submitted by Ponzy Lu and co-workers, which deals with a way to determine the shape of a macromolecular structure (in this case, RNA) through the use of time-resolved fluorescence emission. They convincingly show that this technique can serve as a useful supplement to existing NMR and X-ray methods.

With this edition of the Newsletter, we are starting a new column which we call “Zaps and Stars”. Zaps and Stars is devoted to our experiences, or those of our readers who write in, of products or services that are used in laser based research. Generally, our Stars (thumb up) will reward exceptional quality instruments or service while Zaps (thumb down) will allow us to share with our readership our dissa- tisfactions. If you have any comment which you would like expressed in this forum, please e-mail them to me along with your name and affiliation.

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Are There Identifiable Ultrafast Steps in Protein Folding and Unfolding?

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INTRODUCTION

Questions concerning the physical and chemical nature of protein folding are among the most challenging in biological research. It is not yet understood how proteins proceed from unfolded forms to their stable, biologically active structures. Folding and unfolding events have seldom been studied on time scales shorter than milliseconds. On the other hand, it is well-known that internal motions of macromolecules such as rotations about single bonds, chemical exchange reactions and other barrier crossing processes, can occur on nanosecond or even picosecond time scales, so there is reason to expect that protein structure reorganization will also involve some ultrafast intermediate steps. Studies of the folding of cytochrome-c by monitoring the optical spectra of the heme cofactor have shown already that kinetic changes associated with folding can occur on the 10’s of microseconds time scale. Some of the faster processes in protein folding might involve relatively small alterations in electronic structure. Therefore the probes used to examine them must be sensitive to subtle changes in, for example, the non bonded interactions, the weaker chemical bonds, the charge distributions and motions of pieces of the structure. It is for this reason that we decided to use infrared (IR) spectroscopy to probe the dynamics: It is both structure sensitive at a chemical bond resolution and readily adapted to obtain time resolved kinetics on any desired timescale from fs to ms. The infrared spectra of proteins in the region of the amide vibrations of the polypeptide structures are well-known to be sensitive to the state of the protein. For example, there are distinct differences between the IR spectra of random coil, α-helical, β-sheet, β'-sheet and turn structures of polypeptides. These differences arise from the dependence of interactions between the various amide groups on the local polypeptide structures. One can therefore conceive of carrying out time resolved IR capable of following the kinetics of structure change as it affects these different spatial regions of the polypeptide backbone.

Although the methodologies for transient IR are in place, experiments on the kinetics of folding also require that the system be triggered to suddenly change and that the trigger period be shorter than any kinetic process of interest. For this purpose we have developed an ultrafast temperature jump (T-jump) method (See Newsletter #16) that when combined with transient IR spectroscopy, allows us to measure the protein spectral response to the T-jump as well as the time dependence of the temperature of the medium.

The T-Jump Method

To induce unfolding we use an optically generated heat pulse to raise the temperature of the solvent environment. Lasers have been used for many years to induce temperature jumps in solution, normally on a ns to ms timescale. In absorbing organic media, temperature jumps on the order of tens of degrees can be achieved within 10 ps by means of infrared absorbing dyes which have
picosecond relaxation times and almost unit efficiency for internal conversion to hot ground state molecules. In order to heat protein solutions we have sought water soluble dye transducers, one example of which is the triphenylmethane dye, crystal violet (CV). The dye CV has very efficient internal conversion that leads to rapid heating of its surroundings. It is obviously crucial that the protein and dye are independently and homogeneously dispersed in the solution.

The T-jump was quantitatively analyzed by studies in the absence of protein. When CV was dissolved in aqueous solution (1.5 mM) and pumped by the 532 nm pulse, a reduction in absorbance signal at 2270 cm$^{-1}$ due to the heating of the bulk water (H$_2$O in this example) within the beam volume ($\sim$10$^{-6}$ mL) was observed. Changes in water absorption (shown in Figure 1) occur only within the period of the instrument function of 45 ps. The temperature rise in this experiment is 10±1 K. As discussed below this measured temperature is an average and does not represent a uniform bulk temperature until ca. 70 ps after the energy deposition at the dye concentrations used in the experiment. Experiments on the solutions containing protein are readily carried out in the same manner. The pulse energy (in joules) required to raise the water temperature by 1$^\circ$ at unit density and heat capacity of 1 cal gm$^{-1}$ K$^{-1}$ is 4.2v/$\alpha$ where v is the irradiated volume and $\alpha$ the fraction of incident light absorbed by the dye. In the present case v = 3.5x10$^{-5}$ cm$^3$ and $\alpha$=0.18 yielding 81.7 $\mu$J for the required energy. Thus we expected about a 7$^\circ$ temperature rise for a ca. 500 $\mu$J incident pulse which is close to what was measured.

Protein-Dye Interactions are Absent

There were three specific methods used to test whether the dye Crystal Violet compromised the integrity of the protein. In the first set of experiments with RNase-A, static FTIR spectra of the protein plus dye solution before and after addition of the CV over a period of two hours indicated spectral changes of less than 0.9% of the overall absorbance. The optical transitions of the dye are known to be sensitive to changes of solvent environment therefore the UV/visible spectra of CV in water were compared in the presence and absence of the protein. Peak positions, ratios of peak heights of both the 306 nm and 540 nm bands with and without protein and full widths at half-maximum of the bands were within 0.3% of one another. Thirdly, molecular weight resolution experiments were performed on the RNase-A/dye mixture. A Sephadex G-25 gel filtration column (fractionation range 1000-5000 Da) was prepared in a pH 5.7 MES buffer. A small amount of the protein/dye solution was eluted through the column. Complete recovery of protein occurred within the first 25 mL of elutant with no measurable co-elution of dye. No evidence for any dye-protein association was found in any of these experiments. It follows that CV
and RNase-A are independently dispersed in the solution and that CV is likely to be a useful transducer for some other proteins as well.

The time resolution of this T-jump method is set by three controllable factors: 1) the width of the excitation pulse; 2) the relaxation times of the dye and response of the solvent to heating, and; 3) the time delay in obtaining a uniformly heated solution.

It is known that the absorption coefficient of water at the frequencies used and over the temperature ranges employed in this experiment is essentially linear in the temperature:\textsuperscript{11} It follows that optical density changes in the IR spectrum of the heated water should be independent of the spatial distribution of deposited energy or temperature. That is, the absorbance change of water is not sensitive to the dynamics of the thermal diffusion from the hot spots created by the cooling dye molecules. The laser pulse width of ca. 25 ps is considerably greater than the relaxation time of the dye (< 1 ps) and the spectral response time for impulsively heated water (ca. 4 ps\textsuperscript{11}). After the laser pulse is absorbed, the sample has not necessarily reached an equilibrium temperature. At the concentrations of dye (9x10\textsuperscript{17} cm\textsuperscript{-3}) and protein (4.5x10\textsuperscript{17} cm\textsuperscript{-3}) used in the experiments the mean nearest neighbor distance between any pair of solutes is 50 Å and that between dye molecules is 57 Å. The spatial distribution of heat energy released by each dye molecule should be predictable by classical heat diffusion for times larger than a few tens of picoseconds, so that exp \([\frac{-r^2}{4\chi t}]\) is the ratio of the energy (temperature) found at distance \(r\) from the dye to that at the origin (the dye location) after time \(t\). Thus the time at which this distribution will achieve a variance of (50 Å)\textsuperscript{2} is obtained from \((2\chi t)^{\frac{3}{4}} = 5.0 \times 10^7\) cm, which yields \(t = 113\) ps, where \(\chi\) is the water thermal diffusion coefficient of 1.11 x10\textsuperscript{-3} cm\textsuperscript{2}sec\textsuperscript{-1}. It follows that there is a waiting time until the profile of the heat released by the dye will be contained in a volume large enough to incorporate a protein. If required, the time resolution could be improved by increasing the protein concentration.

A proper assessment of the time resolution of the protein heating experiment requires that all the dye molecule heat sources be considered. An infinite lattice model for the spatial distribution of excited dye molecules in the solution yields an approximation to the bulk medium. With the protein at the origin and dye molecules located at all lattice points on a cubic lattice with cell dimension \(d\), the temperature at the origin is obtained by summing the contributions at each time \(t\) from each dye transducer treated as a point heat source. The lattice spacing was chosen to be the mean value of the distribution of nearest neighbor distances. This temperature (Figure 2) is seen to rise with a time constant of ca. 50 ps and does not change with time beyond ca. 100 ps. Thus we may safely conclude that the protein and its immediate environment will have fully reached the

![Figure 2 Calculation of the time dependence of the temperature at the center of a protein arising from an infinite array of point heat transducers. The dye concentration is 9x10\textsuperscript{17} cm\textsuperscript{-3}.](image-url)
new temperature by ca. 70 ps after the T-jump impulse under the present experimental conditions. We adopt this as the time resolution of the T-jump.

The Change in the IR Spectrum of the Protein Amide I-Region

The first experiment probing the Amide I (C=O stretching) region of RNase-A measured the time dependence of the C=O absorption in a spectral region that is associated with β-sheet structures (1630 cm\(^{-1}\)) following a T-jump from 59-62.5°C. The bleaching signal arising from depletion of β-sheet C=O was the largest overall signal in the static temperature difference IR spectra\(^{12,13}\). The time dependent signal from the protein solution exhibits an initial change due to the change in D\(_2\)O absorbance with temperature that is detailed in Figure 2. In addition to this background signal (See Figure 3) there is a further bleach which is absent if, prior to the T-jump, the solution is more than 10°C below the denaturation temperature. The Amide I regions near 1666 cm\(^{-1}\) that are anticipated to be associated mainly with random coils, bends, α-helix and turns exhibited similar kinetic signals, except that the slow component beyond 1 ns is an increasing absorption signal, consistent with the change expected if the system is tending toward the equilibrium difference IR spectrum. These signals are due to changes in the IR spectra of the protein. The kinetic response corrected for changes in the water spectrum is shown in Figure 4. The 1666 cm\(^{-1}\) absorption increase and the 1630 cm\(^{-1}\) absorption decrease seem to have different time characteristics in this experiment.

In another experiment, a delay of 3.5 ns was maintained between T-jump and probe pulses and the IR probe was tuned to selected wavelengths between 1600 and 1700 cm\(^{-1}\) in order to generate...
the spectrum of the unfolding protein. The data as shown in Figure 5 were corrected for the known D$_2$O background response. Included in the Figure is the difference in the equilibrium IR spectra at the two extremes of temperature involved in the T-jump, recorded at the same concentration as used in the time resolved experiments. The spectrum taken at 3.5 ns and the equilibrium difference spectrum also shown in the Figure, do not show any differences that are yet worthy of detailed discussion given the present signal-to-noise of the experiment.

**Structural and Dynamical Features of the Ultrafast Unfolding**

It is clear from this preliminary study that there are indeed ultrafast changes occurring as the protein unfolds — changes on a timescale that is at least six orders of magnitude faster than that of mixing experiments. Now it remains to try to understand the structural origin of these changes. According to recent studies using low angle X-ray scattering and FTIR$^{12}$ the thermally denatured state of RNase-A is considerably more compact than expected for a Gaussian random coil and contains residual secondary structure. Between $T=58$ and 66 °C the protein undergoes a transition from the native state that has a crystal-like radius of gyration ($R_g$) of 15 Å to denatured state with $R_g = 19.3$ Å, while the $R_g$ for a random coil exceeds 41 Å. Below 61 °C the protein is folded and the (reversible) transition to the unfolded but compact state is complete at 67 °C. This compact, denatured, biologically inactive form has residual secondary structure amounting to as much as 50-67% of the β-sheet found in the native forms.$^{12-14}$

A main feature of our results is that the intensity of the IR band associated mainly with the $\beta$-sheet region (at 1633 cm$^{-1}$) shows ultrafast changes in response to a T-jump that will ultimately convert the stable form of RNase into the compact thermally denatured state. The difference between the spectra of the native and thermally perturbed states at 3.5 ns after the T-jump resembles closely the difference between the equilibrium states at 59 and 65K. This suggests that 30-40% of the population of a structure having amide-I absorption similar to that of the compact denatured state could be present after this time. However, the kinetics are not characterized by a single exponential response to the T-jump: About 1 ns elapsed before any change was detected. This delay is unrelated to the dynamics of the temperature distribution. There is clearly a reduction in the $\beta$-sheet IR absorption at 1630 cm$^{-1}$. One interpretation of these results at 1630 cm$^{-1}$ is that the change in intensity of the amide I absorption corresponds to a reduction in the amount of secondary structure, mainly $\beta$-sheet. Another is that the $\beta$-sheet while remaining intact has undergone structural alterations that significantly modify the IR intensity in the amide-I region. This partial dismantling or perturbation of the $\beta$-sheet appears to involve a cooperative response to the T-jump when observed via the IR intensity of the amide I bands. The occurrence of an induction period during which there is no detectable change in the IR absorption would suggest that critical intermediate configurations need be attained before the spectral changes can occur. These configurations would require to have amide I absorption that is similar to the $\beta$-sheet, and the $\beta$-sheet structure would need to destabilize or become significantly perturbed only after a sufficient number of these configurations is achieved. If the observed change in the $\beta$-sheet IR absorption were to continue toward equilibrium as an exponential process, its time constant would be in the range of 10 ns. In any event it is clear that
the alteration of the β-sheets involves more than one kinetic step. The kinetics we observe are quite different from those reported in chemical mixing experiments that use tyrosine absorption as a probe.\textsuperscript{15}

In that work the first changes detected were on the ms timescale. However, as noted in ref. (15), the time change in the environment of an aromatic chromophore is not a direct measure of all the actual events of unfolding. We can say that significant changes are occurring six orders of magnitude in time sooner than the changes detected in the chemical mixing experiments.

It is unlikely that major changes in the dispositions of sheets, helices, bends and turns could have occurred prior to 1 ns after the T-jump. In any event the protein undergoes a total increase in $R_g$ of only ca. 29\% on denaturation.\textsuperscript{12} However, in order to break up the β-sheet structure it is clearly advantageous that hydrogen bonds be formed with the β-strands: Water should be ideal for this purpose. Thus one structural interpretation of these "critical configurations" is that they arise from water molecules seeping into the hydrophobic β-sheet region because of small structural changes, chain end motion and expansion of the protein induced by the T-jump. They may reach regions that facilitate altering the hydrogen bonds of the β-sheet when appropriate local distortions occur. Water molecules might alter the amide-I absorption without totally disrupting the β-sheets. Heat capacity measurements have indicated that in the unfolded state of RNase-A there is significant hydration of naturally hydrophobic groups.\textsuperscript{16} The importance of water to the early stages of protein unfolding was proposed recently as a result of molecular dynamics simulations on the protein barnase.\textsuperscript{17} This theoretical work demonstrates that water molecules are able to diffuse into hydrophobic regions of barnase (which, unlike RNase-A, does not have disulfide bonds) and dismantle the β-sheet on the ca. 70 ps. time scale. This is considerably faster than the 1 ns delay observed in this work with RNase-A. With improvements in the frequency range and signal/noise of the technique introduced here it should be possible to investigate such intermediate hydrogen bonded states directly.

**Conclusion**

We now have the answer to the question posed in the title: There are indeed identifiable ultrafast steps in the protein unfolding process, at least for RNase-A.

The RLBL is in process now of building a T-jump apparatus and detection system using transient IR that should have considerably better signal/noise and time range than was used in the preliminary experiments discussed here. Hopefully, this new apparatus will be "user friendly" and permit the initiation of some significant collaborative endeavors.

**Acknowledgments**

We are greatly indebted to Professor Walter Englander for his invaluable comments on this research and to Professor Ponzy Lu for his helpful suggestions regarding protein-dye interactions. The research was supported by NIH through RR01348 and GM12592. This paper is taken with permission from an article by CMP, YM and RMH submitted in January 1995 to PNAS.

**References**

Secondary Structure of the r(CUUCGG) Tetraloop

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Introduction

The RNA sequence, r(CUUCGG), occurs often and forms a hairpin (tetraloop) that melts 20° higher than expected. The tetraloop (RNA0) with the sequence r(GGACUUCGGUCC) has been observed to adopt a hairpin or tetraloop conformation by solution NMR and a double helical conformation with central base pair mismatches by X-ray diffraction. Why do the solution NMR structure and the single crystal X-ray diffraction structure on the same molecule disagree? We monitored the secondary structure of this RNA molecule using time-resolved Fluorescence Anisotropy (FA) and 19F NMR, as the solution composition was changed from the solution NMR experimental conditions (the common buffer system for nucleic acids, L-buffer: 10mM sodium phosphate, 0.01 mM EDTA, pH 6.5) to those used for crystallization [(D-buffer: 100mM sodium citrate, 50mM Tris, pH 8.5, 2.5mM MgCl₂) + 12-32% polyethylene glycol 400-this polymer is normally used for nucleic acid crystallization].

Experimental

We measure the rotational relaxation times (τrot) of the RNA oligonucleotides by monitoring the fluorescence emission of the dye ethidium bromide (EB) intercalated between base pairs (bp). Fluorescence energy transfer
occurring between two dyes is avoided by working at ratios \([\text{oligo}] / [\text{EB}]\) larger than 10 (in our experiments, \([\text{oligo}] > 50 \mu M\) and \([\text{EB}] < 5 \mu M\)). Figure 1 shows a calibration curve of how the rotational times of eight well-characterized DNA duplexes, corrected for temperature and viscosity, are proportional to their associated number of bp \((N_{\text{bp}})\). All experimental points merging on a single straight line confirms that this FA experiment is dependent only on the hydrodynamics and size of the nucleic acid duplexes.

**Results and Discussion**

The rotational relaxation time \(\tau_{\text{rot}}\) measured for the RNA dodecamer (RNA0) is presented in Table 1. These data show that this oligonucleotide takes a hairpin conformation regardless of solvent conditions. Thus, the differences in solution conditions used for the NMR and X-ray studies are not enough to induce the hairpin to duplex conformation change. The equilibrium \(\text{hairpin} \rightarrow \text{duplex}\) is a bimolecular event which is affected by the hairpin concentration. Because the RNA strand concentration is high in the crystal (180 mM compared to 50 \(\mu M\) in our FA experiments), we tethered the RNA dodecamers with four uridines (RNA3), so that the local strand concentration is greatly enhanced (~50 mM). Since RNA molecules have \(\tau_{\text{rot}}\) longer than same length DNA analogs, the calibration curve, obtained with DNA duplexes, could not be used for RNA molecules. We designed control oligonucleotides that fold into a known conformation for comparison. RNA1 forms a hairpin with a 12 bp stem and a four uridines loop. RNA2 forms two stable hairpins tethered by four uridines. Due to their different conformations, these molecules yield different \(\tau_{\text{rot}}\) which allowed us to assign the conformations of RNA3 by comparison. In both buffers, we find that RNA3 folds into two tethered hairpins. Thus just increasing the local strand concentration is not enough to induce duplex formation.

**Table 1. Hydrodynamics of the RNA tetraloops:**

<table>
<thead>
<tr>
<th></th>
<th>DNA duplex</th>
<th>RNA 0</th>
<th>RNA 1</th>
<th>RNA 3</th>
<th>RNA 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>1.8</td>
<td>5.5</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystal</td>
<td>2.1</td>
<td>6.9</td>
<td>*</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>

Since the RNA strand concentration in the crystal is 180mM, over 2M accompanying cations must be present to
ensure charge neutrality. Such high salt concentrations induce EB to unbind from the oligonucleotide, which makes the fluorescence decay analysis too complicated. In order to characterize the conformations of our RNA molecules at high salt concentration (2.5M NaCl), we turned to $^{19}$F NMR. A $^{19}$F fluorine nucleus was introduced at the uridine 6 of RNA0 and RNA3. Fluoro-uridine 6 is unpaired in the hairpin conformation but is paired in the duplex conformation, thus yielding a measurable chemical shift between the two conformations. At high salt, RNA0 exhibits a single peak characteristic of the fluorine chemical shift of the hairpin alone. However, RNA3 shows two distinct peaks at high salt in both the NMR buffer or the X-ray crystal buffer. One peak is attributed to the hairpin while the other one is attributed to the duplex. Thus two effects are necessary to induce duplex formation: high concentrations of RNA strand and salt. However, all our experiments indicate the hairpin species as being the major one, whereas the duplex only is observed in the X-ray crystal$^2$. We believe that this results from crystal packing forces which induce the duplexes to stack end-to-end in an orderly array.

High concentrations of RNA0 and salt yield a detectable duplex population. Due to crystal packing forces, the duplex species alone crystallizes leading to the observed crystal structure$^2$. Since the majority of nucleic acids in the cell are highly condensed (ribosomes, chromosomes and a variety of particles) the local cation concentration needs to be considered. For example, the *E. Coli* ribosomal subparticles have cation concentrations of greater than 1M after considering the total charge of all the proteins. Rapid methods to assess secondary and tertiary structures as a function of solvent as described here becomes a necessary adjunct to standard three-dimensional approaches of NMR and diffraction analyses.

References:


“ZAPS AND STARS”

🗹 A star to Coherent, Inc. for the speed with which they have responded to service calls during this past year.

🗹 A zap to Precision Instruments for their abysmal record in responding to service calls, returning items sent for repair and generally following up on commitments that should go along with regular business practice.

🗹 A star to the Chemistry Department and the University of Pennsylvania for their quick and willing response to our need for renovations of the Laser Facility.

COLLABORATIVE PUBLICATIONS


Energy transfer between the core antenna proteins and the reaction center of photosystem II. J. DePaula, A. Liefshitz, W. Lin, V. Chopra, S. Williams, S. Betts

CORE PUBLICATIONS

Ultrafast dynamics as seen through the vibrations. R.M. Hochstrasser, in Royal Netherlands Academy Colloquium on Femtosecond Reaction Dynamics RNAS Proceedings (Elsevier, Amsterdam, 1993).


Electronic coupling and conformational barrier crossing of 9,9'-bifluorenyl studied in a supersonic jet. P.G. Smith, S.M.


