The RLBL continues its efforts to develop laser methods and instrumentation for biomedical research. The Core of the research here involves learning how to manipulate infrared pulses in experiments with complex systems, microscopy and single molecule experiments, the development of methods of triggering conformational dynamics, and transient spectroscopic methods in wide spectral and time regimes. We are very interested in having users who propose significant experiments that require our unique approaches. In addition we are always interested in collaborative projects that challenge the instrumentation at its cutting edge. Recently there were a number of exciting new discoveries arising from both the Core and the Collaborative projects.

One advance concerns the 2D-IR spectroscopy method that was outlined in the last Newsletter. We have been able to obtain an accurate description of the 2D spectrum of a model pentapeptide by using its known structure. This was the next step toward realizing the goal of inverting these 2D spectra into peptide structures, and its success makes us optimistic about the applicability of this method.

In a related area, nonlinear IR spectroscopy (in particular, photon echoes) is a technique which can probe structural fluctuations in proteins and peptides. Strides have been made to extend echoes into the mid-IR region where information regarding the dynamics of structural fluctuations are contained within the inhomogeneous lineshape distribution. The feature article of this issue, written by Prof. Hochstrasser, describes in detail how such an experiment is performed and interpreted.

There are currently a number of single molecule experiments underway at the RLBL. By tagging one helix of a GCN-4 peptide with a donor and another helix with an acceptor we monitored uncoiling by observing a change in energy transfer efficiency. These experiments are in collaboration with W. DeGrado of the Biophysics Department. We are also using this technique to monitor single cellular components in vivo (in collaboration with S. Petska of Rutgers) as well as RNA polymerase transcription complexes (with R. Ebright of Rutgers) and ribosomal transcription processes (with B. Cooperman of the Chemistry Department). We investigated the structure and dynamics of single light-harvesting complexes (LH-2) immobilized on mica under physiological conditions. The B800 BChl a of the LH-2 are excited with linear polarized light whose polarization direction is electro-optically modulated. New, very exciting results suggest that the structure of the LH-2 ring under these experimental conditions might not be circular.

Optical triggering of conformational changes in proteins and peptides also remains as a major component to our Core and Collaborative activity. This area involves both optically induced temperature jumps and photolysis of disulfide bonds. The parametric generation of mJ energy nanosecond near-IR pulses has allowed us to generate fast TJump triggers for protein dynamics. We have coupled T-jumps to fluorescence probe techniques in order to monitor changes in mutant proteins that have tryptophan residues in various spots along the protein as well as fluorescently tagged proteins and peptides. In addition, investigations of disulfide linked peptides are continuing. Temperature and solvent effects on helix formation are now being investigated. Simultaneous experiments in both the visible and IR regions will provide complementary data to form a more complete picture about the pathways of α helix formation.

Another instrumental thrust is terahertz (THz) spectroscopy. This area is just now emerging as a spectroscopically useful tool for investigating low frequency motions in biological systems. We routinely generate ps THz pulses through novel ultrafast methods. We are at the moment investigating nonlinear optical effects induced by the THz radiation field. Other experiments involve investigating the low frequency modes involved in conformational dynamics.

If any of our readership would like to evaluate how any of these methods might add a dimension to their research, please contact us. Detailed facility information is listed on page 7. Mark Phillips cmphilli@mail.sas.upenn.edu
FEATURE ARTICLE

Vibrational photon echoes measure protein fluctuations [1]

Robin M. Hochstrasser

Recent developments in IR pulse generation at RLBL have enabled direct measurement of the correlation functions of protein fluctuations.

Introduction

Chemical and conformational reactions in biology can be controlled by the fluctuations in the energies of the reactant states. Motions of the charges in the surrounding medium cause changes in the electric fields in the neighborhood of the reacting species that in turn cause fluctuations of the reactant energies. For chemical processes occurring in a protein environment, such as in enzyme catalysis or electron transfer, these dynamics of the protein nuclei can limit the reaction rate. Therefore it is important to know properties of the fluctuations such as their time correlation functions and structural origins. At present much of this information derives from classical molecular dynamics simulations.

The experimental approaches to determining time correlation functions include spectroscopic line shape measurements and time domain responses. Time domain techniques can measure the various contributions to the line width such as dephasing and population relaxation7. The homogeneous dephasing times of vibrational transitions having static inhomogeneous distributions can be measured by means of two-pulse photon echoes3-5. However, Ippen and coworkers6 showed that a photon echo with three pulses can also yield the dynamics of the inhomogeneous distribution. This idea was used in the study of cofactors in light harvesting complex II7, reaction centers8, and Zn-myoglobin9. Recently we accomplished such an experiment with femtosecond infrared (IR) pulses exciting vibrational modes10,11 and at the same time introduced the idea of using molecular vibrations to probe time dependent changes in the heterogeneity of proteins. In these studies the dynamics of the inhomogeneous distribution of vibrational frequencies is obtained from the time evolution of the vibrational frequency distribution. The vibrational frequency of a bond is changed when its interaction with its surroundings changes the chemical bonding. The idea is quite general: the vibrational transitions could involve bonds that are intrinsic to the protein, or extrinsic, such as molecules or ions bound to protein or enzyme active sites.

The infrared three pulse photon echo method

In the echo experiment, three femtosecond infrared pulses having propagation vectors \( k_1, k_2, \) and \( k_3 \) interact with the sample and generate two new fields into the two directions \( -k_1+k_2+k_3 \) and \( k_1-k_2+k_3 \) as pictured in Figure 1a. The variable pulse sequence \( T, \tau, t \) is depicted in Fig. 1(a) where \( T \) is the time separation between the peaks of pulse 3 and pulse 2 for \( \tau>0 \), or between the peaks of pulse 3 and pulse 1 for \( \tau<0 \). If the first pulse in the echo sequence is the one propagating along \( k_1 \), the echo is emitted into direction \( -k_1+k_2+k_3 \), if it is along \( k_2 \) the echo is seen at \( k_1-k_2+k_3 \). These echo signals are measured as intensity versus \( \tau \) at various values of \( T \). When the vibrators change their frequencies very rapidly compared with the time scale of the observation, the system is said to be homogeneous, and only then will these two signals be identical. Any system will appear homogeneous when \( T \) becomes large enough that there is no longer any memory of the distribution of frequencies that was excited by the first pulse in the
sequence. Therefore it is the \textit{temporal evolution of the inhomogeneity} that is measured in this experiment. The protein interaction with the probe molecule is modeled by the correlation function of the vibrational frequency fluctuations, \( \delta \omega_1(t) \) or \( \delta \omega_2(t) \), about their mean values. These mean values, which are the transition frequencies connecting the probe vibrational quantum states \( v=0 \) or \( v=1 \) and \( v=2 \) are separated by \( \Delta \), the anharmonicity of the vibration.

**Photon echo measurements**

Tunable femtosecond IR pulses having pulse duration 120 fs, energy ca. 1 \( \mu \)J, band width ca. 150 cm\(^{-1} \), and frequency centered to the absorption lines of the \( \text{N}_3^- \) or CO in the different experiments, were generated by mixing the outputs of a BBO-optical parametric amplifier in a AgGaS\(_2\) crystal. The output was split into three beams each with the same polarization and energy 300nJ. Two mercury-cadmium-teitelluride detectors recorded the signals phase matched in the two directions \( \mathbf{k}_1+\mathbf{k}_2+\mathbf{k}_3 \) and \( -\mathbf{k}_1+\mathbf{k}_2+\mathbf{k}_3 \).

In Figure 2, the signal as a function of \( \tau \) for some selected times \( T \) is shown for both directions. Neither signal is symmetric in \( \tau \), however, a careful inspection indicates that the asymmetry of each of the signals with respect to \( \tau \) decreases as \( T \) increases. This is most evident for Hb-CO where the longer vibrational lifetime permits the process to be followed for up to ca. 40 ps. In the case of CA-N\(_3^-\), the frequency correlation function decays over the first 4 ps while for Hb-N\(_3^-\), it stays constant during this period after a small initial decay. The inhomogeneity of Hb-CO decays on at least two time scales within the observation window of 40 ps.
Local structure is probed
The time dependence of the inhomogeneous distribution has a special significance in the case of proteins. The potential energy surface of the probe molecule is changing as a result of the interaction with the protein. The potential is sensitive to forces that influence the bonding in the probe. Therefore, this method is a probe of the local structure. The local forces can be changed either by fluctuations in the local structure, or by the local structure responding to changes in other parts of the protein. This picture suggests a plausible interpretation of the time sequence of events in the evolution of the inhomogeneous distribution around a local region.

The stimulated echo results for the proteins are to be contrasted with those on isolated ions\textsuperscript{10,11} in water where the inhomogeneity disappears after only few ps. For the case of azide in water the relaxation time of 1.5 ps is not that of water\textsuperscript{12} but it corresponds to the making and breaking of the hydrogen bonds between azide ions and water molecules, establishing that it is the local structure fluctuations that dominate the spectral diffusion\textsuperscript{11}.

Fluctuations in the active site of carbonic anhydrase II
Carbonic anhydrase (CA-II) is a zinc enzyme that catalyzes the interconversion of CO\textsubscript{2} and bicarbonate. The azide ion, which is a competitive inhibitor of bicarbonate dehydration in CA-II, binds at Zn\textsuperscript{2+} without compromising the three-dimensional structure of the active site of the enzyme\textsuperscript{13,14}. The active site is a Zn\textsuperscript{2+} ion, buried by ca. 10 Å from the protein surface and coordinated in an approximately tetrahedral geometry to four ligands\textsuperscript{13,14}: azide, His-94, His-96 and His-119. The azide nitrogen (N\textsuperscript{(1)}) closest to the metal and the central nitrogen (N\textsuperscript{(2)}) have short contacts (3.3 Å) to the hydroxyl oxygen of Thr199. In addition, N\textsuperscript{(2)} (at 3.7 Å) and N\textsuperscript{(3)} (at 3.5 Å) must sense the amide nitrogen of Thr-199. Therefore it is natural to invoke the nearby Thr-199 as the group that modifies the potential energy function and controls the charges of Zn-bound azide. The charge on N\textsuperscript{(2)} in isolated azide is ca. +1e, while the end atoms each have a ca. –1e. Calculations have shown that the Zn bound azide N\textsuperscript{(2)} becomes more positive by 0.11e, N\textsuperscript{(1)} becomes less negative by 0.03e and N\textsuperscript{(3)} more negative by 0.24e when the effect of the enzyme environment, mainly the Thr-199, is taken into account\textsuperscript{15}. These simulations and quantum chemical calculations suggest that protein motions should cause fluctuations in the admixtures of the dominant valence bond structures N\textsuperscript{-1}=N\textsuperscript{+1}=N\textsuperscript{-1} and N=N\textsuperscript{+1}.N\textsuperscript{-2} of the bound azide. Each admixture corresponds to a different potential energy function for the ground state and hence a different vibrational frequency. The presence of a small admixture of the triple bonded structure causes the averaged frequency to increase significantly.

Fluctuations of the CO ligands of hemoglobin
Figure 3 shows the stimulated echo arising from the CO vibration at 1951 cm\textsuperscript{-1}. Again there is evidence for a significant local structure evolution over the time scale of the experiment. The correlation function of the frequency fluctuations does not decay exponentially (it was fitted to a sum of two exponentials plus a part that is stationary over the time scale of the observations). The variations in the CO potential can be caused by its interactions through the iron with the heme and its proximal ligand, and by forces exerted on the CO by the amino acids in the distal heme pocket. It is known that the CO vibrational frequency and hence the potential energy for the CO stretching motion, is extremely sensitive to the presence and positioning of His-E7 in
hemoglobin$^{16}$ and myoglobin$^{17,18}$. Variations in the relative positioning of the polar His-E7 and the CO can cause mixing of the valence bond structures $\text{Fe}=$C=O and Fe-C≡O, the latter being stabilized by the hydrogen bond to E7 causing an increase in the vibrational frequency. Again we invoked medium induced mixing of valence bond structures as a model for fluctuating the vibrational frequency. We suggested that the 12 ps process, which is a main part of the correlation function, corresponds to relaxation of these distal pocket structures. To prove this, studies of a wide range of mutants will be needed.

According to our results, motion within the distribution of substates that modify the CO vibrational frequency begins significantly on the time scale of 12 ps. This implies that the CO oscillator potential in Hb-CO is varying significantly over this time scale. However even after the experimental limit of 40 ps the inhomogeneity is not yet equilibrated and its complete relaxation must be stretched over an even longer time scale. The slower changes in the CO potential must require more complex reorganizations of the protein, presumably involving many more protein atoms. The residual inhomogeneity is therefore attributed to more global structural changes. Forces on the iron, transmitted by motions of the proximal structure and heme would fall into this category and would cause changes in the CO frequency but in a less direct manner.

**Fluctuations of the N$_3^-$ ligands of hemoglobin**

Myoglobin azide has a structure with the linear azido group forming an angle of ca. 60° with the heme plane normal$^{19}$. A close polar contact with the ligand is the distal histidine, His-64, but the ligand is in Van der Waals contact with numerous other amino acids in the heme pocket. The lifetime of the azide transition limits the data collection to ca. 4 ps. However it is apparent that the correlation function is very different from that obtained from CO in the same environment. It is decaying significantly more slowly at early times. This suggests that the inhomogeneous distribution is not relaxed by local fluctuations on a fast time scale. It could be that the azide is significantly more tightly bound to sites in the heme pocket than is CO.

**Further developments**

Protein motions are expected to stretch over many orders of magnitude in time. Methods of examining these motions will give results that are dependent on what is probed. A unique feature of the vibrational approach is that small molecular or ionic probes can be employed, thereby allowing information about local structural fluctuations to be obtained. Optical probes are usually fairly
large cofactors or dye molecules. Their responses are generally assumed to provide properties of the charge fluctuations of the whole medium. It is the fluctuations of the parts of the protein that are directly coupled to the probe and are therefore the main perturbers of its potential energy surface, that are important for small molecule vibrational probes. Accurate calculations of these local fluctuations would seem to be achievable, providing a direct link between theory and experiment.

References


SUMMARY OF CURRENT TECHNOLOGICAL DEVELOPMENT AND RESEARCH AT 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 e-mail us. A fuller description of each of these categories can be found on our Web page at http://rlbl.chem.upenn.edu.

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- Dynamics of photoactivatable proteins and other biological structures by time resolved spectroscopy
- Investigations of protein folding and macromolecular conformational dynamics by time resolved optical, fluorescence and IR spectroscopy
- Investigations of single molecular assemblies using confocal and atomic force microscopies
- Energy transfer and fluorescence monitoring of biological dynamics
- Development of time resolved far-IR (terahertz) probes for protein dynamical changes
- Array detection of broadband pulses in the infrared

A SELECTION OF RECENT COLLABORATIVE PUBLICATIONS


A SELECTION OF RECENT CORE PUBLICATIONS


Application for Use of the RLBL

Title:

Keywords (optional)

NIH Axis Numbers (optional)
Axis I
Axis II

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Abstract: Describe briefly (200-250 words) the scientific goals and methods.

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

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