Editorial
By Thomas Troxler

The previous few Issues of our Newsletter mainly reported news of the progress and new developments in our Core research areas. For the current Issue we take the opportunity to introduce some of our collaborative research projects. These collaborative projects represent one of the three pillars of our research along with the Core and Service activities. They use the emerging technologies in major research applications. At the same time they also increase the scope of the applications of advanced laser technology in biology and widen the range of scientific and technological challenges to our laboratory. Due to the variety of experi-

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mental expertise and experimental arrangements that are set up in the RLBL, our environment is an ideal place for such collaborations and many of them have been exceptionally successful.

The first article by Krause et al. is a collaboration with the University of Medicine and Dentistry of New Jersey and describes recent results about the ligand-induced changes of an interferon-γ receptor complex. These studies involved fluorescence microscopy on living cells using single and two photon excitation and fluorescence resonance energy transfer techniques. Direct measurements of distances between interferon-g receptor chains before and after engagement of ligand suggest a new paradigm for receptor structure and function. The second article by Mei et al., a collaboration with the Biophysics Department here at the University of Pennsylvania, reports on findings about single molecule phosphorescence spectroscopy, especially the measurement of a distribution function of oxygen quenching rates. These studies help to address the blinking of fluorescence of single molecules by studying directly the phosphorescence emission. Following this article is a third report by Fang et al. in collaboration with scientists at Mount Holyoke College, which applies our core research technique of two-dimensional infrared spectroscopy to the study of vibrational interactions within an α-helix. Synthetic peptides were labeled with both $^{13}$C=16O and $^{13}$C=18O in the secondary structure to establish common patterns in the two-dimensional IR spectra. We hope that these research reports show the wide scope of projects that are being carried out at the RLBL.

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We would like to encourage you to visit our website at http://rlbl.chem.upenn.edu. An extended description of the resources is available to outside users, as is information about new instrumentation when it becomes available for collaborative or service research projects. Please don’t hesitate to contact us if you are interested in any of the instruments and approaches to discuss possible collaborative and service projects, or if you have any other questions concerning our activities. ttroxler@sas.upenn.edu

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the constituent proteins, and delineation of the signal transduction events. All these results were deduced from indirect measurements. These models implied that the receptor complex is assembled by ligand. However, more recent observations have begun to suggest that receptor chains can be preassociated in the cell membrane.

To provide a direct way to clarify the various models of receptor action, we established an approach to measure interactions of receptor chains in living cells. Focusing on the IFN-γ receptor we used fluorescence resonance energy transfer (FRET) to assess the structure of the receptor chains and the effects of ligands directly.

Indirect evidence exists about the structure of the IFN-γ receptor complex prior to its activation by IFN-γ. Accordingly, most models of the IFN-γ receptor consider it to consist of four chains: two IFN-γR1 and two IFN-γR2 chains. The ligand IFN-γ is a dimer that binds to the two IFN-γR1 chains. Upon IFN-γR1 binding the receptor chains associate to form a condensed complex. But very little is directly known about the physical association of the receptor chains of such multiprotein complexes and whether chains from one receptor type directly interact with another.

FRET is a powerful spectroscopic technique that utilizes the strong dependence of the energy transfer efficiency on the distance between the donor and acceptor.
tor moiety. For example, this technique has been used to determine protein-protein interactions (4) or the distance between two parts of the ribosome (5). Because it is a noninvasive technique, in-vivo measurements of acceptor/donor interactions are easily possible under physiological conditions. Here we report our results of FRET in examining the IFN-γ receptor structure directly and provide evidence for a new model of its structure and function.

Materials and Methods

To carry out our FRET studies, IFN-γR1 and IFN-γR2 chains with EBFP (enhanced blue fluorescent protein, the donor) and GFP (green fluorescent protein, the acceptor) fused to the carboxyl termini of their intracellular domains were constructed (Figure 1). Because it was necessary to have cells express two proteins labeled with EBFP and GFP at similar levels, we used a single vector expressing both proteins for transfection rather than co-transfection with two vectors. Thus, tandem vectors, in which transcription of each cDNA is controlled by its own separate promoter and polyadenylation signal on a single plasmid, were constructed as described previously (6).

To demonstrate GFP and EBFP fluorescence and FRET, a confocal microscope was modified to include a monochromator associated with a back-illuminated liquid nitrogen-cooled charge-coupled device (CCD) camera so that fluorescence emission spectra and images of whole cells could be obtained from the illuminated cells. The S65T variant of GFP with an excitation maximum at 488 nm was used in all our studies (7). Single-photon excitation at 488 nm of the GFP with an argon laser delivering 0.5 μW at the sample yielded the signature GFP emission having a maximum at 509 nm. The BFP and EBFP have excitation and emission maxima at 380 nm and 445 nm, respectively. Because we found that single-photon excitation of the cells at 380 nm produced very high background fluorescence, we used two-photon excitation of at 760 nm to substantially reduce the sample excitation volume along the quartz cover slides. This resulted in significantly decreased background fluorescence. The 760 nm light was produced by a femtosecond mode-locked Ti:sapphire laser. Typical laser power in this case was 2 mW. We found little or no cellular damage due to the two-photon beam compared with ultraviolet excitation. If FRET occurs between EBFP and GFP, then the emission maximum of GFP at 509 nm will be observed instead of the pumped EBFP emission at 445 nm.

The constructs expressing IFN-γR2/GFP and IFN-γR2/EBFP were individually transfected into COS-1 cells, and images were taken with a camera attached to a confocal microscope. The images show that both IFN-γR2/GFP (Fig. 2, left) and IFN-γR2/EBFP (Fig. 2, right) were visualized by epifluorescence after transfection. Similar images were obtained for the labeled IFN-γR1 samples (not shown). To further demonstrate that the constructs were functional when transfected together into cells, a tandem vector coexpressing IFN-γR2/GFP and IFN-γR1/EBFP was transfected into Chinese hamster ovary cells expressing none of the human IFN-γ receptor chains (CHO q3). The MHC Class I surface antigen induction in response to IFN-γ in CHO q3 cells expressing IFN-γR2/GFP and IFN-γR1/EBFP showed that both receptor chains are functional. Similar activity measurements with other receptor chains such as IFN-γR2/EGFP, IFN-γR2/EBFP, and FL-IL-10R2/GFP demonstrated that all these receptor chains with fluorescent proteins fused to their carboxyl termini were fully functional.

Results and Discussion

Transient transfectants in COS-1 cells expressing matched (Hu-IFN-γR1 and Hu-IFN-γR2) and mismatched (Hu-IFN-γR1 and Hu-IL-10R2) receptor pairs were prepared as shown in Figure 3. The matched pair, Hu-IFN-γR1/EBFP and Hu-IFN-γR2/GFP, represents the two chains of the Hu-IFN-γ receptor com-
plex. The mismatched pair represents two chains from similar receptor complexes, Hu-IFN-γR1/EBFP and Hu-IL-10R2/GFP, which were not expected to interact.

FRET was measured upon excitation at 760 nm in cells expressing each of these pairs of receptor chains in the absence of ligand, given in Figure 4. The spectrum of cells expressing the mismatched pair Hu-IFN-γR1/EBFP and Hu-IL-10R2/GFP shows the EBFP emission spectrum with its peak around 450 nm together with background fluorescence. The minuscule amount of GFP fluorescence due to energy transfer demonstrates little or no interaction of these receptor chains. In contrast, the matched pair, Hu-IFN-γR1/EBFP and Hu-IFN-γR2/GFP, excited again at 760 nm, exhibits the typical fluorescence emission signature of the GFP (peak around 510 nm), demonstrating clearly that energy transfer occurs after excitation between the EBFP and GFP proteins. The calculated distance between the intracellular regions of IFN-γR1/EBFP and IFN-γR2/GFP chains from these data was (5) 36 Å. Furthermore, these spectra revealed that IFN-γR1 and IFN-γR2 chains are preassociated prior to ligand binding as no ligand (IFN-γ) was added yet. Subsequent addition of the ligand IFN-γ to cells expressing the mismatched pair IFN-γR1/EBFP and IL-10R2/GFP did not affect the spectrum (not shown): the spectra were found to be basically identical in the presence or absence of IFN-γ. In contrast, the effect of adding IFN-γ to the matched receptor pair expressed in cells showed that IFN-γ produced a change in the emission spectrum, causing a major reduction in the FRET compared with the FRET in the absence of IFN-γ (Figure 5). The distance between the intracellular regions of Hu-IFN-γR1/EBFP and Hu-IFN-γR2/GFP chains in the presence of the ligand IFN-γ was calculated to be 63 Å, almost twice as large as the 36 Å determined in the case where no IFN-γ ligand was present. Therefore, the results strongly indicate that the intracellular domains of the IFN-γR1/EBFP and IFN-γR2/GFP chains move apart on addition of the ligand IFN-γ.

Our results further demonstrate directly that the receptor chains of the IFN-γ receptor complex are preassembled on the cell membrane of intact cells. Furthermore, the data prove that the receptor complex consists of four (or possibly more chains) because both the IFN-γR1 and IFN-γR2 pair (Figures 4 & 5) and the IFN-γR2 and IFN-γR2 pair (Figure 6) are preasso-
associated. It also became evident from our data that the IFN-γR2 and IFN-γR2 chains are preassociated even in the absence of the IFN-γR1 chain, but that the presence of the IFN-γR1 brings the two IFN-γR2 chains closer together. However, IFN-γ shows no effect on the separation distance of IFN-γR2 chains when IFN-γR1 is not present. This is in agreement with previously published reports, where it was found that IFN-γ does not bind to the IFN-γR2 chain and that IFN-γ exhibits no activity in the presence of only the IFN-γR2 chain only (8, 9, 10). In the presence of IFN-γ-R1 however the efficiency of FRET between two IFN-γR2 chains decreases upon addition of IFN-γ to cells.

Thus the interaction of IFN-γ with its receptor complex also results in the spreading of the intercellular domains of the two IFN-γR2 chains. We suggest that this paradigm is likely to be applicable to other receptor chains and that receptor chains are preassociated in cells ready for activation by ligand. This conclusion leads to the deduction that cell surface receptor chains have specific sites, receptor association regions, that enable the receptor chains to preassociate. Moreover, the increase in distance between the intracellular regions of the receptor chains after the addition of ligand to cells opens the receptor complexes for the attachment of the signal transduction components that are excluded from the receptor complexes until ligand engagement. Thus, our direct measurements of the distances between the IFN-γ receptor chains before and after engagement of ligand suggest a new paradigm for receptor structure and function.

General comments and conclusions

Previous techniques used to measure protein-protein interactions often removed the proteins from the context of their superstructure within membranes and other cellular components with which they are an intricately associated. Thus, high affinity interactions, but not low affinity interactions, can be measured by these procedures. In contrast, as we showed in our work, the use of FRET in combination with microscopy permits the direct visualization of all, low and high affinity, interactions in cells. Association constants that are too weak to be observed in solution after disruption of cells can be clearly seen by the FRET technique (Figs. 4, 5, and 6) because the receptors remain anchored in the membrane.

Because FRET is a real-time technique, changes in protein-protein interactions or protein positions in complexes within living cells can be determined over the periods of time or under changing conditions of the system. Continued use of the FRET technique should provide answers to questions about association of specific receptor chains of multichain complexes, and whether chains from one receptor type directly interact with those from another type. Furthermore, such studies can be extended to the downstream signal transduction events by determining the order, interactions, and kinetics of these processes.

Literature

Direct Observation of Triplet Emission of Single Molecules and Phosphorescence Quenching by Molecular Oxygen

By E. Mei, R. M. Hochstrasser and S. Vinogradov

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Introduction

Newly emerged room temperature single molecule spectroscopy has opened a way for studying heterogeneity present in materials and bio-macromolecules, as well as the dynamics of entities like proteins and enzymes. By studying molecules one at a time, one can probe chemical dynamics and kinetics and obtain direct distributions of physical and chemical properties. Such distributions are not directly accessible by conventional ensemble methods. So far a wide range of single molecule detection technologies based upon different detection schemes has been explored. Among them, single molecule spectroscopy based on fluorescence detection has proven to be most powerful and effective. Its advantages include high sensitivity, low noise level and easy applicability in bio-systems.

In the time-intensity records of these fluorescence experiments, so-called “blinking”, when the fluorescence signal fluctuates between states of high and very low emission intensity, is often observed. This blinking can interfere with the data interpretation, especially if there are other dynamics present that can cause a rapid change in emission efficiency, which will cause similar on/off characteristics of the fluorescence signal (1,2). Although the blinking process has been studied extensively, no detailed and generally applicable mechanism has been established so far (3,4).

In this work, we address the problem of blinking by studying not the fluorescence but the phosphorescence emission. Since the triplet state is the lowest excited state of the molecule, this study should enable us to gain insight into the underlying photo-physical and photo-chemical dynamics, which are obscured in the case of fluorescence detection. Furthermore, using oxygen as a triplet quencher, we are able to obtain unprecedented information about the detailed mechanism of phosphorescence quenching at the single molecule level. Quenching of phosphorescence has also been applied to biological studies on a molecular scale. For example, oxygen quenching of tryptophan (5,6) and metalloporphyrin (7) phosphorescence has been used to evaluate pathways of oxygen diffusion inside proteins and look into the dynamics of oxygen channels.

Materials and Methods

We employed two molecules, PtOBCP (= Pt octabutoxycarbony porphyrin) and Ru(ddp)3Cl2 (= Ru(II)-tris-4,7-diphenyl-1,10-phenanthroline dichloride) for our studies. Absorption and emission spectra of PtOBCP in deoxygenated DMF are shown in Figure 1. The phosphorescence lifetime is 36 µs in the absence of
oxygen. Samples for single molecule imaging and spectroscopy were prepared by spin casting of a 1% PMMA solution onto a quartz cover slip. Typical sample concentrations were 0.1 nM. Bulk phosphorescence decay time measurements in solutions or PMMA films were performed using previously described methods (7). Single molecule experiments were performed with an inverted confocal microscope and Argon ion laser as an excitation source. A liquid nitrogen cooled CCD detector and an imaging spectrograph were coupled to the microscope and used to record the single molecule phosphorescence spectra. Oxygen concentration of the samples was adjusted using an inverted glass funnel connected to gas tanks of nitrogen, oxygen and argon. A system of multiple inlets and flow meters allowed for gas mixtures to be varied in the desired proportions.

Results and Discussion

Figure 2 compares single molecule phosphorescence spectra of PtOBCP and Ru(ddp)3Cl2 with the corresponding bulk spectra. Phosphorescence was observed in the same region and with the same wavelength characteristics in both cases. Adding oxygen to the sample clearly quenches the phosphorescence emission as shown in Figure 3. In these experiments single molecules were first observed under a pure Argon atmosphere. Oxygen was then added and additional images of the same region were taken.

The relationship between oxygen concentration n(O2) and triplet lifetime t is usually described in bulk solutions by the Stern-Volmer equation

\[
\frac{I_0}{I} = \frac{\tau}{\tau_0} = 1 + K_Q n(O_2)
\]

where I0 and \(\tau_0\) are the intensity and phosphorescence lifetime in the absence of oxygen, \(k_2\) is the bimolecular rate constant for the quenching and \(K_Q\) is the related constant. This relationship was also found to be valid in the case of our single molecule images. A linear relationship should be observed between the intensity ratio and the oxygen concentration. The quenching constant itself will depend on the diffusion rate of O2 to the phosphorescent and on the mechanism of quenching within the encounter complex. Usually it is assumed that quenching occurs via a short range energy exchange, resulting in the production of singlet oxygen.

Single molecules of Ru(ddp)3Cl2:

Individual quenching events could be measured in the case of triplet quenching of Ru(ddp)3Cl2 by oxygen. This permitted an experimental evaluation of the distribution of quenching constants that are hidden from ensemble average measurements in a typical bulk experiment. Figure 8 shows such an experimentally determined distribution of quenching constants. The mean bimolecular rate constant \(k_2\) is equal to 2.2x10^8 l mol^{-1}s^{-1}, which corresponds to a mean equilibrium quenching constant \(K_Q\) of 1120 l mol^{-1}. The distribution function is not well fit by a gaussian function and it clearly stretches out to larger quenching constants. This suggests that there is a range of accessibilities to oxygen that is very likely related to the distance distribution of Ru(ddp)3Cl2 molecules from the surface of the film. Reported quenching rate constants for similar Ru(II) complexes in solutions range from 2.2 - 4.2x10^9 l mol^{-1}s^{-1} [41]. Thus it appears that on average the solubility factor of Ru(ddp)3Cl2 in the surface layer is about 0.1.
We could further show that PtOBCP could be employed a single molecule oxygen sensor. This result could be useful for further studies of heme protein dynamics because PtOBCP can be incorporated into various proteins. Such an ultra-small sensor could be crucial for non-invasive intracellular oxygen detection and diffusion rate determinations. There is a need in biochemical and medical research to measure oxygen concentrations in living cells or tissues. In principle, our studies suggest that both PtOBCP and Ru(ddp)3Cl2 offer this possibility by employing time gated methods to discriminate between the fast scattered signal and the slower phosphorescence.

**Fluorescence Blinking**

It is very common to observe large intensity jumps in the fluorescence signal of single molecules on time scales that are long compared with ordinary photophysical parameters. Sudden changes in the orientation of the emitting dipole can affect the overall emission signal strength, but in the two cases presented here, PtOBCP and Ru(ddp)3Cl2 are immobilized in a PMMA film. The observed dark states most likely correspond to reversible chemical transformations whereby the chemistry might involve oxygen, the polymer matrix or both. To provide trapping times on the order of tens of seconds, escape barriers would require to be in excess of 10 kcal/mol. One possibility involves the formation of long lived Pt porphyin cation radicals. Such involvement of cation radicals was previously suggested in the case of single molecule studies of light harvesting complexes LH2 (8). It also has been shown that ZnTPP cation radicals can survive in some confined molecular environment for minutes (9). Nevertheless, further experiments are required to establish with certainty if the recovery of the phosphorescence signal from such dark states after tens of seconds is a thermal or photo-induced process.

**Conclusions**

In summary, we have demonstrated the successful observation and characterization of single molecule phosphorescence and the experimental determination of oxygen quenching constants. Based on our experiments, a single molecular oxygen sensor employing either Ru(ddp)3Cl2 or PtOBCP seems to be feasible. This would allow for the non-invasive measurement of
oxygen concentration in cell and tissues.

Acknowledgement

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Literature


TWO DIMENSIONAL INFRARED MEASUREMENTS OF THE COUPLING BETWEEN AMIDE MODES OF AN α-Helix

By C. Fang, W. Barber-Armstrong, R. M. Hochstrasser and S. Decatur

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Introduction

The development of methods to obtain structural changes in proteins is an important experimental challenge to which two-dimensional infrared spectroscopy (2D IR), developed at the RLBL, can contribute significantly. The development of 2D IR started with the observation of infrared photon echo in the early seventies followed by two pulse echoes in the visible. As optical lasers improved, investigations into faster responding liquids and glasses became feasible and new techniques like heterodyning, gating and three pulse echoes were successfully introduced. With the current availability of femtosecond laser technology heterodyning of phase-locked IR echoes and dual-frequency IR pump-probe experiments are suitable to measure 2D IR spectra of peptides and small proteins. All these techniques open up the exciting possibility of studying protein dynamics in real time over several orders of magnitudes through a detailed analysis of vibrational mode coupling in the multidimensional IR spectrum.

IR spectroscopy provides the fingerprints of nuclear motion in any molecule. Within proteins, motions of each peptide unit give rise to characteristic vibrational frequencies of which the amide I band around 1600-1650 cm⁻¹ is the most intense. According to normal mode calculations, its main contribution comes from the C=O stretch. The amide I band also shows structure sensitivity due to frequency shifts from exter-
nal/internal hydrogen bonding and electrostatic coupling to neighboring units. Utilization of linear IR spectra to deduce structures of proteins and peptides has been limited because of the congestion from overlapping amide I bands associated with different structural domains. Methods based on nonlinear IR spectroscopy overcome these shortcomings by spreading the spectrum into more frequency dimensions. 2D IR spectroscopy has emerged as a powerful tool to investigate structural dynamics not accessible in a linear approach.

Materials and Spectroscopic Results

25-residue helical peptides based on Ac-(A)₄K(A)₄ - K(A)₄Y-NH₂ [6] containing the isotopic amide carbonyl labels ^13^C=^16^O and ^13^C=^18^O were obtained by standard peptide synthesis, purified by reverse-phase HPLC and characterized by electrospray mass spectrometry. The ^18^O amino acids were synthesized by ^18^OH₂ exchange. The isotopomers contain none, one or one of each of the isotopic amide carbonyl labels and are referred to as [0,0], [0,11], [12,13], [11,13] and [11,14]. The numbers refer to the isotopically labeled amino acids in the chain. With this set of isotopomers we were able to distinguish between direct neighbor and non-direct neighbor interactions.

Figure 2 shows linear IR spectra of the four isotopomers [0,11], [12,13], [11,13] and [11,14] in the region of the amide I band obtained with a conventional FTIR instrument at room temperature. A baseline spectra consisting of a sample cell with solvent but no peptide was subtracted in all cases to obtain these spectra. The thick spectral line shape was fitted to four gaussian peaks for each case corresponding to the main helical ^12^C=^16^O peak at 1632 cm⁻¹, a weaker non-helical ^12^C=^18^O contribution at 1655 cm⁻¹, the ^13^C=^16^O isotopomer helical peak at 1594 cm⁻¹ and the ^13^C=^18^O isotopomer helical peak at 1571 cm⁻¹. The peak separations between the two isotopic labels in the [12,13], [11,13] and [11,14] isotopomers were 24.7, 22.4 and 28 cm⁻¹. Diagonal anharmonicities, i.e. the change in frequency of a single vibration as its excitation number increases, for both isotopically substituted amides were determined by broad-band pump/probe experiments [1] and were in the range of 12-15 cm⁻¹ for all isotopomers.

Nonlinear 2D IR spectra of the same isotopomers were obtained precisely as in previous heterodyned echo experiments at 6 μm [2-5]. Three infrared pulses with wave vectors k₁, k₂ and k₃ were incident on the sample. Either k₁ (rephasing spectrum) or k₂ (non-rephasing spectrum) arrives first, followed with a time delay τ simultaneously by k₂/k₃ or k₁/k₃. The echo signal at wave vector –k₁+k₂+k₃ was observed with a fourth heterodyning laser with wave vector k₄ at a delay time τ after the second excitation pulse sequence. Scanning the two delays τ and t and Fourier transforming the resulting two-dimensional sample response yields the 2D IR spectrum along the frequency axes ωτ and ωt. For example, Figure 3a shows the (absolute value) 2D IR rephasing spectrum of the [11,13] isotopomer and Figure 3b corresponding absorptive spectrum. Absolute value thereby means that the real and imaginary part of the Fourier transform were squared and added, and the absorptive spectrum consists of an addition of the real parts of the 2D IR spectrum along the frequency dimension. For example, Figure 3a shows the (absolute value) 2D IR rephasing spectrum of the [11,13] isotopomer and Figure 3b corresponding absorptive spectrum. Absolute value thereby means that the real and imaginary part of the Fourier transform were squared and added, and the absorptive spectrum consists of an addition of the real parts of the rephasing and non-rephasing spectra.

Like the linear FTIR spectrum, the 2D IR spectrum in Figure 3a reveals three main peaks along the diagonal. The intensities are different and more pronounced, because the 2D IR spectrum scales with the fourth power of the transition dipole moment, and contrary to the linear spectrum, the 2D IR spectrum is background free. In addition to the diagonal peaks,
there are several off-diagonal features clearly visible in the 2D spectrum. These off-diagonal peaks are coupling peaks between the two corresponding diagonal peaks in analogy to a 2D NMR spectrum. The cross peaks, also observed in the case of the other isotopomers not shown here, reveal that the isotopomer residues are coupled in all cases. No such coupling could be extracted from the linear spectrum.

In the absorptive 2D IR spectrum shown in Figure 3b again for the [11,13] isotopomer, the anharmonicities of all vibrations are easily revealed. Along the diagonal, “double” peaks are due to the 0→1 and 1→2 transitions. The displacement of these transitions reveals the diagonal anharmonicity of the corresponding vibrations. Off-diagonal anharmonicities between different vibrations within the peptide molecule are again revealed in the many cross peaks identifiable in this spectrum. They are on the order of 3 - 9 cm⁻¹ depending on the isotopomer.

In order to estimate the coupling constants from these measured off-diagonal and diagonal anharmonicities, a model is needed which will be able to reproduce both the linear and nonlinear spectra. Such a model is described in more detail in reference 1. Briefly, coupling constants $\beta_{ij}$ needed to generate the observed set of two fundamentals, two overtones and one combination band were $\beta_{ij}[12,13] = 9.5$ cm⁻¹ for the isotopomer with nearest neighbor isotopic substitution and $\beta_{ij}[11,13] = \beta_{ij}[11,14] = -0.5$ cm⁻¹ for the isotopomers with one or two residues between the substituted sites.

In summary, the insertion of $^{13}$C=16O and $^{13}$C=18O labels at known residues on the $\alpha$-helix enabled the measurement of the coupling between the selected amide-I vibrational modes using a 2D IR approach. The observed signal strength indicates that one in 35 residues could have been detected, meeting the requirement for application of the 2D IR method to single residue specificity in small proteins.

Acknowledgement
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Literature
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.

- **Two-dimensional infrared spectroscopy (2D-IR) and infrared analogues of NMR.** Heterodyned photon echo spectroscopy and spectrally resolved three pulse IR photon echoes are employed to investigate the amide I, amide II and amide A region and other transitions of peptides and small proteins. One and two color experiments are feasible to obtain quantitative information on the anharmonic potential surfaces, the vibrational mode coupling, the frequency distributions and vibrational frequency correlation functions with the highest time resolution. Efforts are under way to couple the 2D-IR technique with temperature jump methods.

- **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 in combination with transient absorption techniques in the visible and infrared spectral regions.

- **Investigations of single molecular assemblies using confocal and atomic force microscopes.** It is now possible to examine the properties of single molecules using fluorescence and phosphorescence in association with confocal microscopy. Studies using resonance fluorescence energy transfer, dual wavelength detection, polarization resolution, two photon absorption are feasible for single molecule investigations of biomolecules and imaging techniques of single cells. The RLBL also has coupled single molecule detection methods with mature time correlated photon counting technology for fluorescence lifetime imaging with polarization resolution. Excitation wavelengths available cover the whole visible range from 400 – 850 nm and include continuous wave and pulsed picosecond and femtosecond lasers. Additionally, an objective-type total internal reflection microscope (TIRFM) that images single molecules by means of a CCD camera has recently been set up and coupled to various laser sources. Typical penetration depth of the evanescent field is ~150 nm, ideally suited to study single molecules located in cell membranes.

- **Dynamics of photoactivatable proteins and other biological structures.** Methods are being used and further developed to examine the responses of biological systems to light by pump/probe and nonlinear spectroscopic techniques 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.

- **Energy transfer and fluorescence monitoring of biological dynamics.** Monitoring fluorescence lifetimes and anisotropies reveals details of structural dynamics of peptides and proteins. Techniques have been developed at the RLBL to monitor these properties of fluorspecies on the femtosecond to nanosecond timescale. Vibrational energy dynamics, electron transfer reactions, and UV cross-linking are open for investigation.

- **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 to access spectral information in the far IR region.
A Selection of Recent Publications


Vibrational dynamics, mode coupling and structure of acetylproline-NH2 dipeptide. I. V. Rubtsov and R. M. Hochstrasser, in Trends in Optics and Photonics, 72, 386 (2002).


Application for Use of the RLBL

Title:

Keywords (optional)

NIH Axis Numbers (optional)
Axis I
Axis II

Investigators (PI first)  Degree  Department / Institution / Address
1.  
2.  
3.  

NIH Support Sources  NIH Start/End Date  Other Support Sources
Grant Number(s)  (MM/DD/YY-MM/DD/YY)  Agency and Grant Number(s)
1.  
2.  

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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