Editorial

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The current issue of our Newsletter concentrates once again on the ever-expanding field of multidimensional spectroscopy and its application to biophysical and biomedical research. We are reporting in our Feature Article recent advances in two-dimensional infrared (2D IR) spectroscopy currently worked on in our laboratories. Within the last couple of years we have built and extended several instruments for multidimensional infrared spectroscopy experiments, which now form a major part of the research capabilities of our Resource, and which are also used in conjunction with outside collaborators. As described in detail in our Feature Article, 2D IR spectroscopy has become a tool to study the structure and dynamics of peptides and proteins. The structural sensitivity of this technique was further increased by the incorporation of isotopic labels. The

Feature Article

RECENT ADVANCES IN 2D-IR

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Introduction

It was not long ago that the extension of nuclear magnetic resonance (NMR) spectroscopy into multiple dimensions [1] was introduced as one of the great advances in the study of the structure of proteins in solutions. Now, these principles of multidimensional spectroscopy have been transferred to the infrared spectrum, where the wavelengths are about 10^-6 of those in the NMR regime, allowing a direct measurement of vibrational couplings combined with the dynamics of excitations transferring between molecular vibrations in peptides and proteins.

The dimension of a spectroscopy refers to the number of independently variable time intervals between the field pulses that induce the signals. For example, with three pulses there are three time intervals of interest — that between the first and second (τ, the coherence time); that between the second and third (T, the waiting time); and the interval (t, the detection time) between the third pulse and the detected photon echo field emitted by the sample in response to all three pulses. The three-dimensional grid of experimental data points obtained by varying these time intervals then defines a response in the time domain which can readily be converted to a three-dimensional spectrum in the frequen-
combination of the structural sensitivity of 2D IR and multiple isotopic labeling creates a very powerful technique that allows for specific access to study interactions between particular sites within a peptide or protein chain. We believe that this technology will have a major experimental impact in structural biology.

Several recent articles published in this newsletter dealt with the application of fluorescence resonance energy transfer (FRET) studies in single molecule research. With our current Research Article starting on page 8 we would like to show once again the wide applicability of this technique, but this time we focus on a study using an ensemble sample. Professor Krishnaswamy from the Childrens' Hospital in Philadelphia is interested in the study of blood coagulation enzymes, specifically mechanisms of substrate specificity of the enzyme complex prothrombinase, and how the physical biochemistry of the interactions that stabilize the complex relates to specific enzymic functions. Together with Professor Boskovic (now at Lola Linda University) they performed a FRET study about the recognition of substrate and product by prothrombinase. The results of their study contrasted with the long standing doctrine that active site-dependent substrate docking drives both affinity and specificity for serine proteases. Their work, part of which was done at the Resource, also introduced me to the fascinating biochemistry of the blood coagulation cascade, a great example of the combination of specificity, response speed, and feedback regulation used in and adapted for the requirement of life processes. I was very happy that I had a chance to be involved in that fascinating work.

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classes $\omega_1$, $\omega_T$ and $\omega_b$ by Fourier transformation along the three time axes. The unique aspect of such spectra is that they exhibit cross peaks at spectral points $(\omega_1 = \omega_A , \omega_T = \omega_B)$ linking two transitions between molecular energy levels at the frequencies $\omega_A$ and $\omega_B$. These cross peaks are only present because the structural components A and B of the system are able to sense one another’s presence: in other words, A and B are close enough to be coupled and the pulse sequence must be inducing transitions between them. This information is not exposed directly by spectra in one dimension.

Multidimensional spectroscopy showing coupling between different structural units has also been demonstrated for electron paramagnetic resonance [2] spectroscopy, for which the wavelengths used are much smaller than in NMR but still in the centimeter range, where the phase is comparatively easy to control. Experiments analogous to those in NMR have been carried out for the IR region of the spectrum [3] at wavelengths of 6 and 3µm, enabling the coupling between molecular vibrations to be exposed. Two-dimensional IR spectroscopy has now become an active field of theoretical [4] and experimental research with applications in unraveling the dynamic structures of peptides and proteins [5-8] and liquids [9].

These experiments clearly reveal which vibrations are coupled, but also yield the time-scales of the energy flow between them and between them and the surrounding medium. This experimental method, because of its relatively broad bandwidth, is not restricted to measurements of equilibrium dynamics. The pulse sequences that generate the photon echo can be applied to a system that has been kicked into a nonequilibrium state by another laser pulse. The new two-dimensional IR methods may be used as structural probes of the kinetics of chemical changes over a wide range of time-scales dictated only by the ability to introduce spatial or electronic delays between short laser pulses; in comparison, the range over which kinetic data can be acquired with NMR is limited at present to tens of milliseconds [10]. The distance scale of the vibrational; couplings ranges up to ca. 1 nm which is similar to NMR.
We hazard a guess that these 2D approaches will ultimately replace conventional infrared spectroscopy (and maybe even ultraviolet, visible spectroscopy) because they contain not only the structural information of the conventional spectra but they tell whether or not the pieces of the structure that are responsible for the absorption bands are in proximity.

The 2D-IR experiments first focused on the structure sensitive amide I region of the IR spectrum of peptides. Recent technical advances have allowed for the coverage of much more of the IR spectral range, and multi-frequency experiments, analogous to heteronuclear NMR, became feasible. Multi-frequency experiments can access structural constraints such as distances and angular relations between different functional groups in the same molecule, like the carbonyl and amine groups of a protein, or in different molecules such as hydrogen bonded pairs or tertiary interactions in proteins.

In the following we describe further recent progress in the application of the 2D-IR method to structural investigations of peptides. In the three sections we present recent technical and/or experimental advances as currently being achieved at the Resource. We focus thereby on the application of isotopic substitutions, our efforts to probe structural changes of proteins in a trehalose matrix, and experiments and simulations of model peptides that help us gain a deeper understanding of the 2D-IR method in combination with the simulation techniques used.

2D IR of α-helix isotopomers

The IR spectra of proteins and peptides are intimately connected with their complete 3D structures on the length scale of chemical bonds, but in practice the vibrational transitions of proteins are not all spectrally resolved. Multidimensional nonlinear infrared methods provide additional information and can be used to measure whether, and by how much, the modes are coupled. Structural constraints obtained from these nonlinear experiments, combined with established concepts of chemical connectivity, are sufficient to establish underlying features of 3D structures. However the intrinsic spectral widths of the vibrational transitions and the small frequency separations between many of the modes make the inversion of spectral data to structure a formidable challenge.

The strategy we recently adopted at the Resource is to use two isotopic labels, $^{13}$C=O and $^{13}$C=O, to better separate vibrational transitions in the protein IR bands. For example we substituted two amide units in the center of a 25-residue alanine rich α-helix of the sequence Ac-(A)₄K(A)₄K(A)₄K(A)₄K(A)₄Y-NH₂, effectively dragging their amide I' transitions out of the main helix exciton band formed by the remaining unlabeled carbonyls.

In this study we were mostly interested in cross-peaks between the two isotopically labeled amide I' modes, which were separated by one, two or three residues. The use of double isotopical substitution helped us to measure directly the vibrational coupling between the two labeled residues placed at different positions within the peptide chain. Because those labels can be inserted into various positions of the helix chain, the 3D structure of the molecule can be revealed, as reflected in the distances between the modes and relative orientations of the pairs of transition dipoles.

Figure 1 shows a view of the residues within the α-helix structure. The view is along the helix axis and the oxygen atoms of amide carbonyl groups are numbered. The carbonyl groups are all approximately parallel to the helix axis, and the nearest neighbor inter-
actions do not involve much intervening polarizable medium. A series of doubly isotopically labeled helices \{12,13\}, \{11,13\} and \{11,14\} ([alanine residue with \(^{13}\text{C}=^{16}\text{O}\), alanine residue with \(^{13}\text{C}=^{18}\text{O}\}) were studied by linear-IR and 2D IR, and some very interesting features have been revealed. For example we identified coupling parameters between each pair of isotopomer vibrational levels and between them and the helix exciton band states: the 2D IR spectra proved that the amide vibrations of the \(\alpha\)-helix are delocalized (e.g. spectrum (a) in Fig. 2). By carefully analyzing each absorptive spectrum (e.g. spectrum (b) in Fig. 2), quantitative information about the inter-residue anharmonicity was obtained: \(\Delta_{12,13} = 9.0\pm1.0\), \(\Delta_{11,13} = 3.2\pm0.8\), and \(\Delta_{11,14} = 4.5\pm0.6\text{ cm}^{-1}\).

The experimental results could be compared in detail with a full matrix diagonalization simulation based on averaged Hamiltonian matrices that represent the amide I' vibrators' one- and two-exciton states. Hence the complexity of interamide coupling and the sensitivity of the coupling to the polypeptide conformation were tested. The main features of the 2D IR spectra could be predicted by this modeling. Figure 2 shows the comparisons between (a) and (a’) for the absolute magnitude rephasing spectra of sample \{12,13\}. Furthermore, the observed experimental frequencies and anharmonicities were in good agreement with a set of coupling constants that were derived from transition charge-transition charge interactions for all but the nearest neighbors, for which the coupling was more influenced by through bond interactions between the adjacent amide groups. The possible ranges of the magnitudes of the three largest coupling constants \(\beta_{12}\), \(\beta_{13}\) and \(\beta_{14}\) were found to be: \(|\beta_{12}|=8.5\pm1.8\), \(|\beta_{13}|=5.4\pm1.0\text{ and }|\beta_{14}|=6.6\pm0.8\text{ cm}^{-1}\). Meanwhile the signs of these coupling constants appeared to be very well determined by the relative transition intensity data either in linear-IR or 2D IR spectra [12]. The readout of these signs was much easier in 2D IR since it’s a background-free technique. The \(^{13}\text{C}=^{16}\text{O}\) transition was significantly stronger than \(^{13}\text{C}=^{18}\text{O}\) in \{12,13\} (manifested in spectra (a) and (b) in Fig. 2), in contrary to the cases in \{11,13\} and \{11,14\} where the \(^{13}\text{C}=^{18}\text{O}\) transition was stronger, the signs were therefore indicated to be \(\beta_{12}>0\), \(\beta_{13}<0\) and \(\beta_{14}<0\). Thinking in terms of the spatial dependence of the interaction energy between two interacting transition dipoles, the experimentally determined signs of the coupling constants agree well with the structure as shown in Figure 1.

Besides structural determination, the 2D IR method is also capable of detecting equilibrium conformational dynamics. The observed dynamics can be related to the breaking and making of hydrogen bonds. All these experiments further strengthen the case that isotopic substitution in combination with 2D IR spectroscopy is a powerful tool to extract structural and dynamic in-

**Figure 2:** 2D IR absolute magnitude rephasing spectrum (a) of \{12,13\}, with two amide labels adjacent to each other. The corresponding simulationed spectrum is shown to the right (a’). See text for more details.
formation at the single residue level.

2D-IR Spectra of Dipeptides in Trehalose Glass

Trehalose is a disaccharide generated in large quantities under desiccative conditions by organisms which undergo anhydrobiosis, or the temporary suspension of biological activity. When the extreme conditions have passed, these organisms are able to resume their lives without any residual damage. Trehalose is an important part of anhydrobiosis due to the high glass-transition temperatures of its partially aqueous solutions. Trehalose glasses have also been proposed as non-damaging media for cryological storage of biological samples due to their presenting a static, “water-like” environment to protein solutes; several studies have been performed to examine the properties of proteins embedded in this matrix [13]. However, the exact nature of the contacts formed between the glassy medium and peptide chains is unclear. 2D-IR experiments were recently formulated at the Resource to explore the structure and dynamics of small peptides in trehalose glasses.

Three model dipeptides were immersed in trehalose/D²O glass, and nonlinear infrared spectra were collected via the three-pulse heterodyned photon echo technique in the amide-I (C=O stretching) region. Infrared correlation spectra for AcProNHMe (A), Ac*AlaNHMe (B) (where Ac* denotes Me¹³C=O) and trialanine-D⁺ (C) are shown in Figure 3. A striking facet of each of these spectra is that the diagonal peaks are inhomogeneously broadened along the diagonal, suggesting a large structural distribution for each dipeptide in the glass. Cross peaks are weak in each spectrum, most likely due to interfering peaks from different structural sub-populations. Compared to similar spectra in D₂O, these spectra exhibit a lack of motional narrowing and possibly a broader structural distribution than in water for peptides in trehalose glass.

Variation of waiting times displayed the lack of time evolution in the spectra. Not only are peptide structural distributions in trehalose very broad, they are apparently fixed on any time scale accessible to our measurements. From the point of view of these measurements, trehalose certainly presents a static structural environment to peptide solutes.

Figure 3: Two-dimensional infrared correlation spectra for AcProNHMe (A), Ac*AlaNHMe (B), and trialanineD⁺ (C).

The nonspecific nature of the hydrogen bond contacts from the glassy solvent leads to large dipeptide structural distributions, which broaden the spectra along the diagonal and obscure neighboring spectral features. An advantage of two-dimensional spectroscopy is that contributions from different mechanisms of line-
broadening are separated from each other along the two spectral dimensions. In the case of trialanine (Figure 3C), cross peaks between the two overlapping amide I bands are obscured in the broad correlation spectrum, but a skew projection of the spectrum (Figure 4) along the diagonal to remove the inhomogeneous broadening can show the presence of these cross peaks. The \( v=0-1 \) cross peaks are visible as small positive signals centered at +20 and -20 cm\(^{-1}\), and their anharmonically shifted \( v=1-2 \) counterparts are negative signals at ca. +10 (overlapped with diagonal signal) and -30 cm\(^{-1}\). The cross peaks are broad and diffuse in this projection, indicating that they originate from a diverse ensemble of structures.

Furthermore, polarization dependencies of these cross peaks enabled us to establish an estimate of the angle between the two C=O transition dipoles of +35-45 degrees, which is different from similar measurement made for trialanine in D\(_2\)O [14]. This suggests a difference in the mean structure for a small peptide in trehalose glass vs. in water.

Through variations of photon echo vibrational spectroscopy, trehalose glass has been shown to be a structurally static environment with its own distinct set of properties at the single-residue peptide level. This work represents an nice application of de facto standard techniques available in the RLBL nonlinear infrared spectroscopy resource.

**Figure 4:** Diagonal skew projection of vertically polarized (<zzzz>) and horizontally polarized (<zxxx>) spectra for Ala3D+.

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**Alanine dipeptide structure determination**

The linear infrared and 2D IR spectra in the amide-I' region of the alanine dipeptide and its \(^{13}\)C isotopomers in aqueous solution (D\(_2\)O) were studied. The alanine dipeptide exhibits two amide-I' IR transitions that have been assigned unambiguously by using \(^{13}\)C isotopic substitution at one of the carbonyl groups. The amide unit at the acetyl-end shows a lower transition frequency in the unlabeled species. The ratio of their transition dipole strengths remains almost unchanged upon \(^{13}\)C substitution, indicating the absence of intensity transfer between the two vibrators. Using the 2D IR technique, 2D IR cross peaks directly associated with intramode coupling between these two carbonyl

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**Figure 5:** Real part of absorptive 2D IR spectra of the isotopomer I and II. Waiting times are given in each plot.

The coupling peaks between the two amides in the modes show a small off-diagonal anharmonicity.
(0.2±0.2 cm⁻¹), leading to a small coupling constant (1.5±0.5 cm⁻¹). The measured coupling and the 2D IR spectra in two different polarizations (<zzzz> and <zxxz>) are as expected for a polyproline-II (PPⅡ)-like conformation for dialanine, with the backbone dihedral angles (φ,ψ) determined to be in the range of 70°±25°, +120°±25°).

The coupling peaks between the two amides in the molecule are not evident in the spectra of isotopomer I (acetyl-end ¹³C labeled), but for II (amino-end ¹³C labeled), for which the frequencies are closer. These real spectra show a very small but definitive coupling peak. The absorptive spectra are shown in Figure 7 at the same two representative waiting times. These absorptive spectra are obtained by adding the real rephasing and non-rephasing spectra at each frequency |ωτ|. The individual rephasing and non-rephasing spectra also provide useful clues regarding the existence of underlying peaks in the spectrum. The correlation spectra indicate a number of features. Again the weak cross peaks in isotopomer II are more apparent than are any cross peaks in I. These results compared with other reported measurements of peptide 2D IR indicate that the coupling between the amide groups is relatively weak in the alanine dipeptide. The other obvious feature is that the diagonal anharmonicities of the two amides are about the same. A detailed analysis shows that they are 15.5 cm⁻¹ and 16.0 cm⁻¹ for the amino-end and acetyl-end, respectively.

The effect of the time interval T is made very clear by the data in Figure 8 which are in the form of contour plots of the absolute magnitude spectra for the isotopomers I and II at T = 0 and T = 2 ps. At T = 0 the peak signals are shifted out to ca. 300 fs along the τ-axis. At later time T the spectra are becoming more symmetric about the axis τ=0. The evolution of this peak position as a function of T is the peak shift. This evolution is also seen in the shapes of the 2D IR absorptive spectra. Figure 8 also shows the effects of interference between the oscillators: the spectra are evidently not simply the sum of those for independent vibrators. The 0→1 and 1→2 parts of the 2D IR spectrum are not resolved in these absolute magnitude spectra, so each sample shows only one peak for the acetyl- and one for the amino-ends of the peptide. Also clear from Figure 8 is that the apparent peak shifts are much larger for isotopomer I (see Figure 8(a)).

Conclusions and outlook

The previous paragraphs showed that we succeeded in measuring the 2D IR spectra of doubly isotopically substituted peptides. Isotopic substitution is a powerful approach because it selectively picks out individual interactions within a peptide or protein for a 2D IR study, allowing for a stepwise approach to decipher molecular interactions and structure. Our analysis of the signal-to-noise level revealed that one isotopic impurity site within a 35-residue chain can be detected. Furthermore, isotopic substitution also helps to enhance a detailed comparison of experimental data with simulated spectra. All studies presented in this Newsletter show the capabilities of the 2D IR technique to reveal site-specific interaction through the measurement of coupling constants and frequency fluctuation correlation functions.

Our work on isotopically substituted peptides is a first step towards the use of isotopic substitution in real proteins in order to disentangle the inherent complexity of these large systems. We currently are involved in collaborative work to apply this technique to β-sheet forming proteins. The technique recently al-
followed for the successful determination of the antiparallel β-sheet structure of a model membrane-embedded peptide [17]. Additionally, we are also involved in investigations of nonequilibrium dynamics initiated by triggering with a T-jump. The combination of 2D IR spectroscopy and external triggering provides a unique approach to address experimentally the nucleation process of secondary structures or rapid barrier crossing processes of small peptides.

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Literature

Research Article

Using the Fluorescence Resonance Energy Transfer Approach to Demonstrate Active Site-independent Recognition of Substrates and Product by Bovine Prothrombinase

By D. S. Boskovic, T. Troxler, and S. Krishnaswamy

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Introduction
Blood coagulation is a life saving mechanism. Within moments of a breach in the vasculature, blood loss is stopped by a series of reactions that culminate in the formation of an insoluble mass that plugs the site of damage. It is important that this process is localized to the site of damage. Through the combination of response speed and specificity, the blood coagulation cascade exemplifies a reaction network that has evolved to fulfill specific requirements that are essential for life.

Damage to the blood vessel initiates blood coagulation by exposing cells bearing tissue factor to flowing blood and by exposure of ligands such as collagen that recruit thrombocytes (platelets) to the fresh wound. Tissue factor exposure leads to the recruitment of VII/VIIa found in blood to the site of damage and initiates a proteolytic cascade. In parallel, receptor-mediated interactions between platelets, von Wilebrand factor and collagen lead to cell adhesion, shape changes and the enabling of other receptor mechanisms all designed to promote the activation of the adhered platelet. These platelet activation reactions are essential to the coagulation process because many of the proteolytic reactions of the cascade are membrane-dependent and require the exposure of phospholipids such as phosphatidylerine on the activated platelet surface to proceed efficiently. The products of the coagulation reactions also play an important role in activating platelets adherent at the site of vascular injury. The interplay between membrane-dependent proteolytic
activation steps and receptor-mediated blood cell activation is essential for amplifying the coagulation response and maintaining the reactions localized to the injury site.

Thrombin is produced by the proteolysis of prothrombin in a final step of the coagulation cascade. Thrombin cleaves fibrinogen to fibrin, which spontaneously polymerizes to form a hydrogen bond mediated fibrous network, that also encloses blood cells to form a hemostatic plug. Thrombin is a pivotal product of the cascade because it acts on a variety of protein substrates to enhance its production, blood cell activation and chemical stabilization of the fibrin clot. In the later stages of the clotting process thrombin plays a negative role by catalyzing the activation of a protease that inactivates cofactor proteins that are essential for reactions upstream in the cascade.

Several of the highly specific activation steps of the blood coagulation cascade are catalyzed by enzyme complexes assembled through protein-protein and protein-membrane interactions involving specific trypsin-like serine proteases and protein cofactor proteins. The prothrombinase complex, that catalyzes the conversion of prothrombin to thrombin, typifies this architecture. Specific recognition and proteolysis of two peptide bonds in prothrombin is achieved by this complex, which comprises the serine protease, factor Xa, the cofactor protein, factor Va, in the presence of a suitable membrane surface and calcium ions [1].

Previously presented kinetic studies indicated that interactions with extended macromolecular recognition sites (exocites), rather than the active site of prothrombinase, are the principal determinants of specificity and binding affinity for substrate or product during the conversion of prothrombin to thrombin. This idea contrasts with the long-standing doctrine that active site-dependent substrate docking drives both affinity and specificity for serine proteinases [2]. Here we report a model-independent evaluation of such concepts by direct binding studies of the interaction of substrate derivatives and product with the prothrombinase complex, using the technique of fluorescent resonance energy transfer (FRET).

Results and Discussion

Materials and experimental procedures are given in detail in reference 3. Purification and characterization of bovine prothrombin, factor Xa, and factor Va followed published procedures [3, and reference therein]. Spectral measurements were performed in assay buffer using a scanning fluorescence plate reader. Fluorescence emission spectra were recorded with a spectrofluorimeter, and lifetime decays were measured using the TCSPC setup of the Resource.

Studies of the individual reactions of prothrombin activation catalyzed by prothrombinase have been assessed using prethrombin-2 (Pre-2) and meizothrombin des fragment 1 (mIIa\(\Delta\)F1) as valid substrate analogues for the two half-reactions of prothrombin activation. Pre-2 modestly quenched the fluorescence intensity of Oregon Green488 covalently tethered via a peptidyl chloromethylketone to the active site of factor Xa within prothrombinase. The observed saturable change in fluorescence intensity is consistent with the ability of this substrate analog to bind the enzyme complex despite an irreversibly blocked active site.

Further work was pursued by the development of a FRET strategy to provide a signal unequivocally related to the binding of substrates and products to active site-blocked prothrombinase. 2,6-dansyl moiety was incorporated into the active site of Xa as fluorescence donor and eosin was incorporated into the active sites of either IIa or mIIa\(\Delta\)F1 as fluorescence acceptor.

**Figure 1:** Fluorescence emission spectra obtained using \(\lambda=330\text{nm}\) excitation for reaction mixtures in assay buffers containing 0.4\(\mu\)M Va, 50\(\mu\)M PCPS, and either 0.2\(\mu\)M Xa\(_D\) plus 1.7\(\mu\)M IIa\(_I\) (for D), 0.2\(\mu\)M Xa, plus 1.7\(\mu\)M IIa\(_E\) (for A), or 0.2\(\mu\)M Xa\(_D\) plus 1.7\(\mu\)M IIa\(_E\) (for D+A).
Emission spectra obtained by excitation of the donor fluorophore in reaction mixtures containing prothrombinase assembled with XaD and IlaE are presented in Figure 1. In comparison with control spectra (D and A), significant donor quenching was obtained in the presence of both donor-labeled prothrombinase and acceptor-labeled thrombin. Minor effects on donor quenching were observed in the absence of Va (not shown), consistent with the greatly reduced affinity for the interaction of substrates or product with Xa in the absence of the cofactor.

Donor quenching increased saturably with increasing concentrations of IlaE (Figure 2). The presence of IlaE shortened the excited state lifetime of the donor (insert of Figure 2). Fractional changes in donor-excited state lifetime with increasing concentrations of acceptor paralleled changes in donor quenching as shown in Figure 2. These observations confirm resonance energy transfer between the donor fluorophore tethered to the active site of Xa within prothrombinase and the acceptor tethered to the active site of thrombin. Analysis of the donor quenching data yielded maximum quenching of 27.6 ± 1.3% at saturation and an equilibrium dissociation constant of 0.9 ± 0.2 µM, which is in agreement with the kinetically determined affinity for this interaction [3]. These data provide independent support for the conclusion derived from the initial velocity studies that thrombin can bind to prothrombinase without engaging the active site of Xa within prothrombinase [4]. Similarly, the FRET study using mIIaΔF1E as the acceptor labeled ligand yielded maximum quenching of 18.0 ± 0.9% and a Keq = 3.0 ± 0.6 µM.

Energy transfer reversibility was demonstrated by a decrease in donor quenching observed with increasing levels of competing unlabeled ligand, such as IlaE (active site blocked but unlabeled thrombin), prothrombin or Pre-2. Increasing concentrations of prothrombin reduced donor quenching observed in reaction mixtures containing IlaE and prothrombinase assembled with XaD [3], suggesting that thrombin and prothrombin bind in a mutually exclusive and active site-independent way to prothrombinase. These observations document for the first time that exosite binding also plays a dominant role in determining substrate affinity for the biological substrate with both cleavage sites intact.

In summary, we have used fluorescence approaches to test a surprising and key prediction that arises from studies assessing the kinetic mechanism of protein substrate recognition by prothrombinase [4]. Equilibrium binding studies now provide direct verification that prothrombin, its derivatives, and thrombin bind to prothrombinase in an active site-independent manner. Our measurements establish a primary role for exosite binding in determining protein substrate affinity and further document that docking of residues flanking the scissile bond in the substrate with the active site of prothrombinase does not contribute in an obvious way to the affinity and binding specificity. These findings challenged existing paradigms while explaining functional diversity in the chymotrypsin family and the narrow and distinctive specificities of the serine proteinases of coagulation.

Acknowledgement
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Literature
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.

- **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. Single or dual frequency 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 either confocal microscopy or total internal reflection fluorescence microscopy:** 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 the fluorescence of single molecules excited by the evanescent generated by total internal reflection by means of a fast 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 for example 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), 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 fluorescing species 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


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