Intramolecular Resonance Dipole−Dipole Interactions in a Profluorescent Protease Substrate

Beverly Z. Packard,*,† Dmitri D. Toptygin,‡ Akira Komoriya,† and Ludwig Brand‡

OncoImmunin, Inc., 335 Paint Branch Drive, College Park, Maryland 20742, and Biology Department, Johns Hopkins University, 3400 N. Charles Street, Baltimore, Maryland 21218

Received: September 2, 1997; In Final Form: November 11, 1997

In this study NorFES, an undecapeptide containing an amino acid sequence recognized by the serine protease elastase, was covalently labeled with two xanthenes, one on each side of its cleavage site, to serve as a tool for examination of intramolecular resonance dipole−dipole interactions. To this end using all possible combinations from the group of xanthenes including fluorescein, tetramethylrhodamine, and rhodamine-X, three heterobichromophoric and three homobichromophoric NorFES derivatives were synthesized; their absorption and fluorescence spectra were measured both before and after cleavage by elastase. In the heterobichromophoric substrates the fluorescence of the fluorophore that would be the nominal donor in a Förster model system was quenched. Since the fluorescence intensity of the nominal acceptor in these substrates was also decreased, these data were not consistent with the Förster model. Rather, spectra for all six doubly labeled peptides could be explained by delocalization of excitation over each substrate's two fluorophores. Thus, by taking into account dipole−dipole interactions between two dyes placed in close proximity to each other, the spectral properties observed could not be ascribed to the monomeric components but were the unique optical signature of each ground-state dimer.

Introduction

Pairs of xanthene dyes, e.g., fluoresceins and rhodamines, have been used for defining distances within and between macromolecules for many years. The interpretation most often applied to these spectral data is based on the Förster model of resonance energy transfer.[1−8] Correct quantitation in the application of Förster's theory is dependent on accurate evaluation of parameters such as the donor's fluorescence lifetime and quantum yield in the absence of the acceptor, the overlap integral between donor and acceptor, and κ^2, the orientation factor between the two chromophores. While most discord revolves around the methodology used in calculating the latter, other aspects of applying the Förster equation to spectral data can also be tenuous. For example NorFES is an undecapeptide which we recently synthesized and derivatized with two different rhodamines believed to be a good Förster pair; however, by comparing the spectral data of this heterodoubly labeled substrate with those of relevant singly as well as homodoubly labeled substrates we found the intramolecular dipole−dipole interaction between the two dyes to be well-described not by Förster but by exciton theory.[9−11]

In this work we extend our previous studies to dipole−dipole interactions between fluorescein and each of the rhodamines. A careful study of these dyes in a simple well-defined molecular system such as the NorFES peptide seemed particularly appropriate at this time due to the extensive use of fluorescein and rhodamines in past as well as ongoing studies throughout the scientific literature. Thus, three sets of xanthene dye pairs often used in molecular dynamics studies where the Förster mechanism is used to interpret the data were covalently bound to the NorFES peptide; dipole−dipole interactions in six bichromophoric, i.e., three heterodoubly and three homodoubly labeled, peptides were compared. Description of the absorption and fluorescence properties of these doubly labeled substrates required analysis of the data which took into account properties lacking in the individual dye components but present in the composite systems.

Materials and Methods

Materials. N_α-9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Calbiochem-Novabiochem (La Jolla, CA). 2-Chlorotrityl resin was obtained from Peptides International (Louisville, KY). The coupling reagent benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was bought from Advanced Chemtech (Louisville, KY). Solvents such as HPLC grade dichloromethane (DCM), methanol, and acetonitrile were from Fisher Scientific Co. (Pittsburgh, PA). Other reagents such as 1-methyl-pyrrolidinone (NMP), 4-methylmorpholine (NMM), 1-hydroxybenzotriazole (HOBT), diisopropylethylamine (DIEA), and protein sequencing grade trifluoroacetic acid (TFA) were from Aldrich Chemical Co. (Milwaukee, WI). Fluorophores 5′,6′-carboxytetramethylrhodamine succinimidyl ester, 5′,6′-carboxy-X-rhodamine succinimidyl ester, and 5′,6′-carboxyfluorescein succinimidyl ester were from Molecular Probes (Eugene, OR), and porcine pancreatic elastase from Sigma Chemical Co. (St. Louis, MO). A C18 reverse-phase HPLC column was from Vydac, Inc. (Hesperia, CA).

Methods. Peptide Synthesis. The reagents and methods used for peptide synthesis and derivatization have been described in detail previously.[9] Briefly, peptides (NorFES sequence = DAIPN$_l$SIPKGY (N$_l$ norleucine)) were synthesized using manual Fmoc chemistry and were sequentially derivatized with the succinimidyl ester of each fluorophore. Crude as well as conju-

* Corresponding author. Phone: 301-314-5959. Fax: 301-314-3636. E-mail: oncoim@access.digex.net.
† OncoImmunin, Inc.
‡ Johns Hopkins University.

S1089-5647(97)02845-9 CCC: $15.00
Published on Web 01/22/1998

gated peptides were purified by reverse-phase HPLC including separation of two fluorophore isomers, i.e., the 5′-carboxyl and 6′-carboxyl fluorophore-derived peptides, at each step.

Digestion of Peptides with Elastase. Enzymatic digestion of peptides by elastase was carried out in a buffer composed of 50 mM Tris and 12 mM calcium chloride, pH 9 at 37 °C.

Spectroscopy. *Absorption Spectra*. All absorption measurements were made with a Shimadzu UV 160U spectrophotometer at room temperature.

Fluorometry. All fluorescence measurements were carried out at 37 °C with an SLM 48000 S spectrofluorometer (SLM-Aminco, Rochester, NY). Excitation was with a xenon arc lamp through a monochromator and a polarizer set at 54.7° to the vertical (the magic angle). Emission was observed through a monochromator and a vertical polarizer. Both monochromators had a resolution of 4 nm full-width at half-maximum.

Calculation of Transition Dipole Magnitudes. The squares of the calculated transition dipole moduli, i.e, $|d|^2$, were calculated by the following equation

$$|d|^2 = \frac{3hcn}{8\pi^3} \int_{\nu_{\min}}^{\nu_{\max}} \frac{\sigma(\nu)}{\nu} \, d\nu \qquad (1)$$

where h is Planck's constant, c is the speed of light, n is the refractive index, σ is the natural extinction coefficient expressed in CGS units, ν is the wavenumber ($\nu = 1/\lambda$), and the wavenumber range ($\nu_{\min}$, $\nu_{\max}$) must completely cover the spectral band corresponding to one electronic transition. In the work presented here integration was over the wavelength range of the lowest energy transition, i.e., in the visible range. Unit conversion from the decadic extinction coefficient ϵ in cm^{-1} mol^{-1} L to the natural extinction coefficient σ in cm^2 is given by the following relation

$$\sigma = \frac{10^3 \ln(10)}{N_A} \epsilon \qquad (2)$$

where N_A is Avogadro's number.

Results

The overall objective of this study was to increase understanding of the dipole−dipole interactions between two fluorophores within the same molecule by measuring the spectral properties of bichromophoric peptides both before and after separation of the dyes. The latter was achieved by addition of the serine protease elastase which recognizes a cleavage sequence in the backbone of the undecapeptide NorFES (DAIPN$_l$SIPKGY (N_l = norleucine)).[9] We were particularly interested in examining the dye−dye interactions of fluorophores often used as Förster pairs. Thus, with carboxyfluorescein (Fl), carboxytetramethylrhodamine (TMR), and carboxyrhodamine-X (RX), we have synthesized and studied the absorption and fluorescence excitation and emission spectra of three heterodoubly labeled and three homodoubly labeled derivatives of this peptide.

Using the two amino functionalities of NorFES, i.e., the α-amino of the aspartic acid and the ϵ-amino of the lysine, three heterodoubly labeled substrates (Fl-NorFES-TMR, TMR-NorFES-RX, and Fl-NorFES-RX) were made by sequentially covalently linking two different fluorophores on opposite sides of the peptide's cleavage site and the homodoubly labeled substrates (Fl-NorFES−Fl, TMR-NorFES-TMR, and RX-NorFES-RX) by linking the same fluorophores. As an aid, singly labeled NorFES derivatives (Fl-NorFES, NorFES−Fl, TMR-NorFES, NorFES-TMR, RX-NorFES, and NorFES-RX) were also synthesized and studied in the same manner.

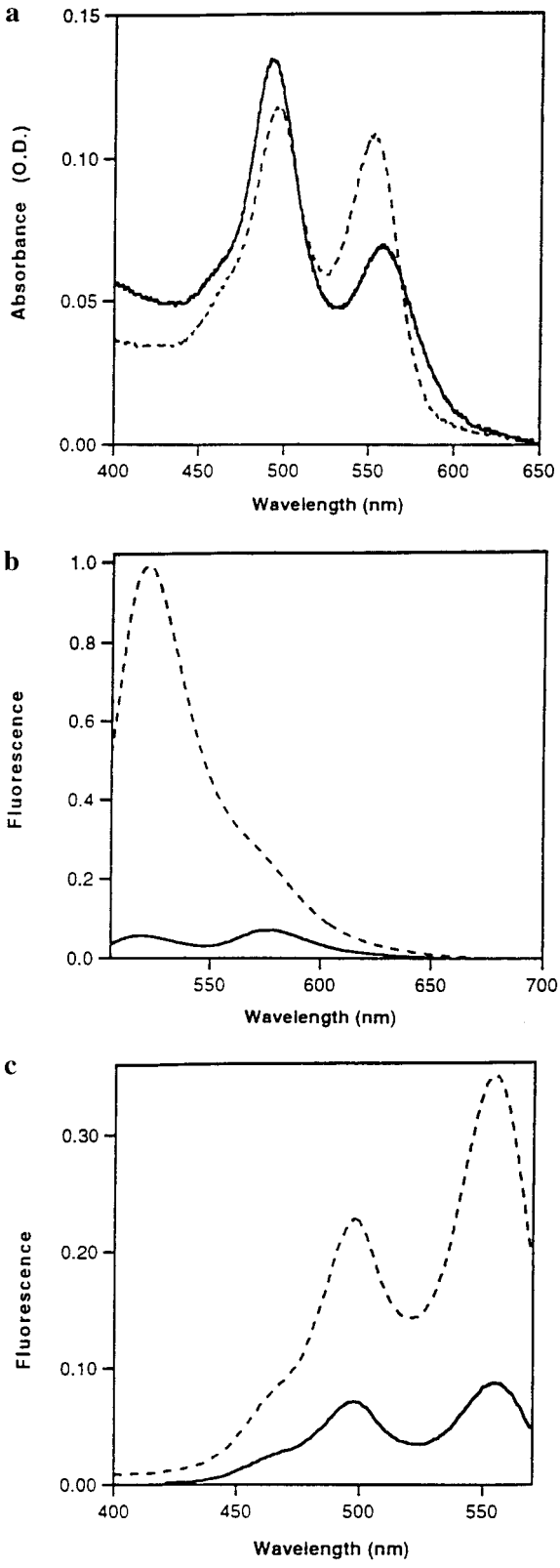

Figure 1. Spectra of Fl-NorFES-TMR (Fl would be the "donor" and TMR the "acceptor" if a Förster mechanism were in effect) before (solid line) and after (dashed line) addition of elastase: (a) absorption spectra, (b) emission spectra (λ_{ex} = 495 nm), and (c) excitation spectra (λ_{em} = 580 nm).

Spectra of D-NorFES-A Derivatives. Spectra of Fl-NorFES-TMR, TMR-NorFES-RX, and Fl-NorFES-RX are

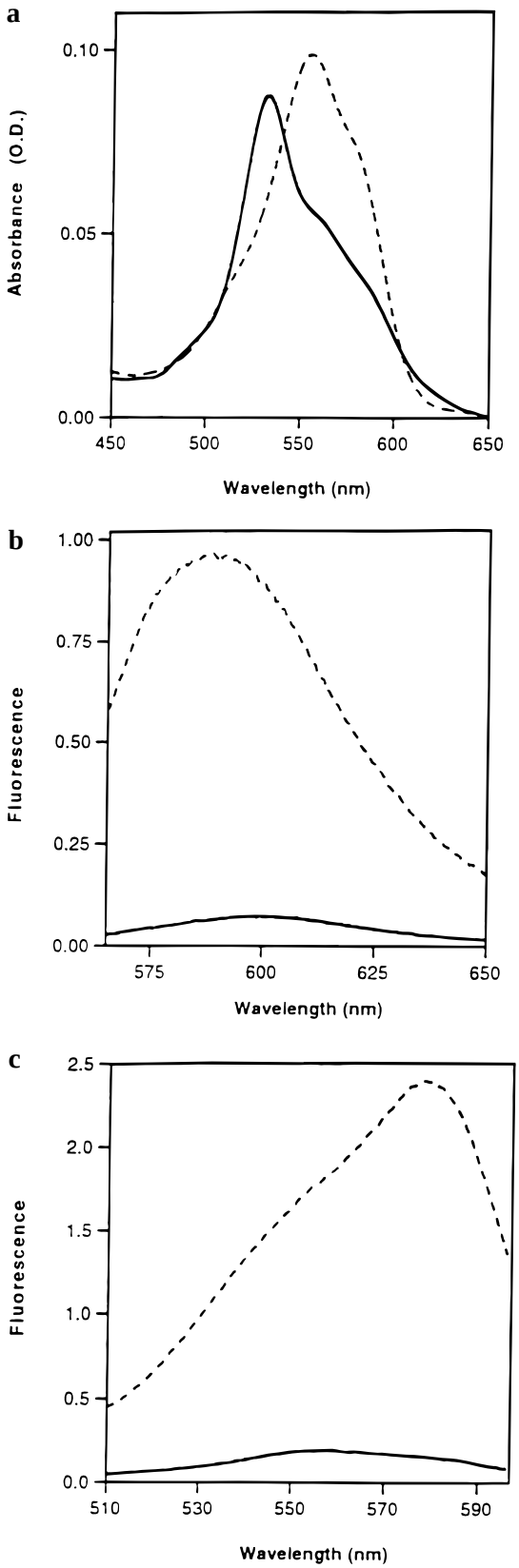

Figure 2. Spectra of TMR-NorFES-RX (TMR would be the "donor" and RX the "acceptor" if a Förster mechanism were in effect) before (solid line) and after (dashed line) addition of elastase: (a) absorption spectra, (b) emission spectra ($\lambda_{ex} = 552$ nm), and (c) excitation spectra ($\lambda_{em} = 607$ nm).

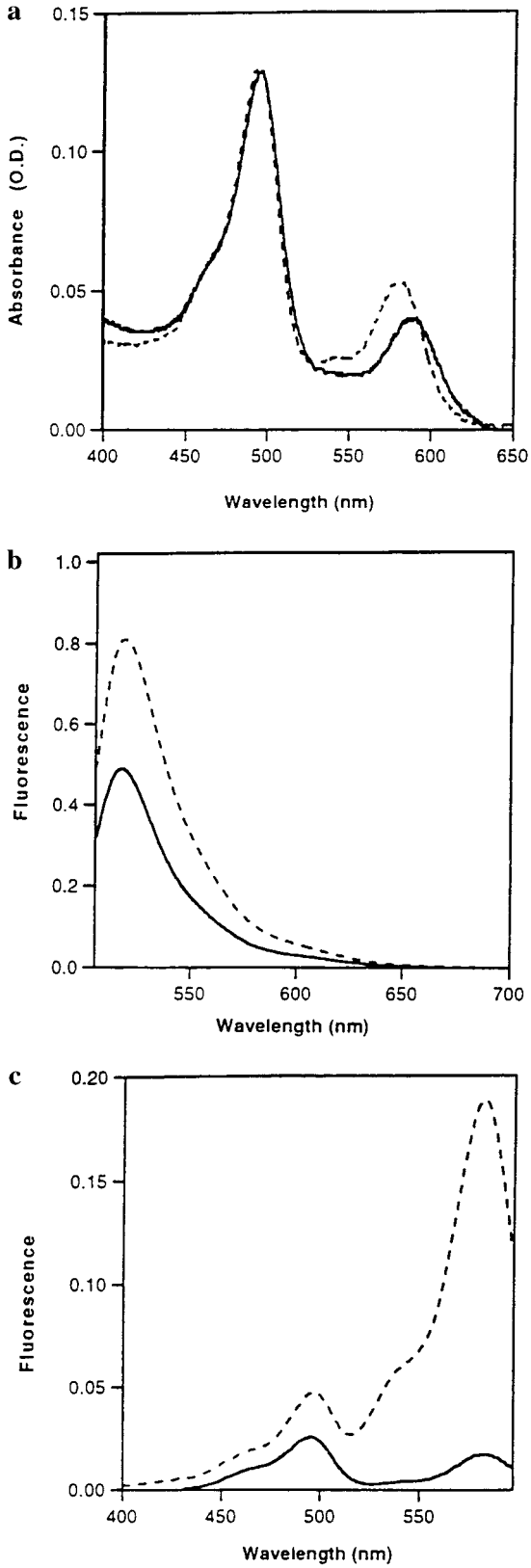

Figure 3. Spectra of Fl-NorFES-RX (Fl would be the "donor" and RX the "acceptor" if a Förster mechanism were in effect) before (solid line) and after (dashed line) addition of elastase: (a) absorption spectra, (b) emission spectra ($\lambda_{ex} = 495$ nm), and (c) excitation spectra ($\lambda_{em} = 607$ nm).

presented in Figures 1−3, respectively, with the a panels showing absorption, b panels emission, and c panels excitation spectra. Solid lines represent spectra of solutions containing the intact peptides and dashed lines the solutions after complete cleavage of the substrate. (Conditions for complete cleavage were previously worked out by HPLC monitoring.[9]) For each emission spectrum the excitation wavelength used was the

TABLE 1

fluorophore	$\|d\|^2 \times 10^{35}$ (erg cm^3)	λ_{ex} (nm)	λ_{em} (nm)
fluorescein (Fl)	7.62	495	520
tetramethylrhodamine (TMR)	9.87	552	580
rhodamine-X (RX)	6.53	580	607

maximum of the fluorophore that would be the donor in a Förster pair, i.e., fluorescein (λ_{ex} = 495 nm) or tetramethylrhodamine (λ_{ex} = 552 nm). For each excitation spectrum the emission was monitored at the maximum of the fluorophore that would be the acceptor in a Förster pair, i.e., tetramethylrhodamine (λ_{em} = 580 nm) or rhodamine-X (λ_{em} = 607 nm) (Table 1).

In Figure 1a one observes in the absorption spectrum of the intact Fl-NorFES-TMR peptide, relative to that of the cleaved peptide, a slight (13.5%) increase in intensity at 495 nm (the peak of fluorescein) and both an intensity decrease (36%) and slight (ca. 5 nm) red shift in the peak at 552 nm (peak of tetramethylrhodamine). Excitation of the intact peptide at 495 nm resulted in an emission spectrum (solid line in Figure 1b) in which two peaks were observed, the first at 520 nm, the peak of fluorescein's emission, and the second at 580 nm, the peak of tetramethylrhodamine's emission. The height of the latter exceeded the former by 23%. Upon complete cleavage of this peptide the spectrum indicated by the dashed line in Figure 1b was recorded; here the intensity at 520 nm increased ca. 17-fold while that at 580 nm increased slightly more than 3-fold. If the Förster model were an accurate descriptor of the spectral characteristics of Fl-NorFES-TMR, then the intensity at 580 nm would have to decrease upon cleavage. Moreover, a comparison of the excitation spectra before and after addition of elastase, monitoring at the emission at 580 nm (Figure 1c), indicates the fluorescence of the putative Förster acceptor, tetramethylrhodamine, is quenched by 75% in the intact peptide.

In Figure 2a the absorption spectra of TMR-NorFES-RX are presented before (solid line) and after (dashed line) addition of elastase. The peak of the former is slightly diminished (by ca. 8%) and blue-shifted (by 20 nm) relative to the latter. These spectral characteristics are indicative of formation of an H-type dimer in the intact peptide. Figure 2b illustrates upon excitation at 552 nm the presence of a ca. 17-fold quenching and a 17 nm red shift in the solution containing the intact peptide relative to that of the cleaved. However, a peak at neither component's emission maximum, i.e., 580 or 607 nm, is observed. Interestingly, observation of an excitation spectrum at the nominal acceptor's emission peak, i.e., at 607 nm, indicates the fluorescence of this fluorophore is quenched to a greater extent than that of the donor. This is also inconsistent with Förster theory.

Data of intact Fl-NorFES-RX plotted in Figure 3a indicate a slight red shift and a decrease in extinction coefficient of the peak ascribed to the RX (λ_{max} = 580 nm), relative to the solution of the cleaved peptide, whereas the Fl peak (λ_{max} = 495 nm) is unaffected. Additionally, upon excitation into fluorescein (at 495 nm), the nominal Förster donor, emission of the two fluorophores is decreased by 41% (λ_{max} = 520 nm) and 45% (λ_{max} = 607 nm), respectively. Monitoring emission at the nominal acceptor's peak, i.e., 607 nm, shows the acceptor's quenching to be more than 2-fold greater than the donor's (91 vs 41%).

Some of the spectral data from Figures 1−3 are present in Table 2. Specifically, the percent quenching (%Q) in the intact peptide relative to the cleaved peptide is shown for excitation at the donor's maximum and emission at the donor's maximum, excitation at the donor's maximum and emission at the acceptor's maximum, and excitation at the acceptor's maximum and emission at the acceptor's maximum. Additionally, the percent quenching for each of the homodoubly labeled NorFES peptides[10] is presented; here, excitation and emission are at the single fluorophores' maxima.

TABLE 2

	λ_{ex}	λ_{em}	%Q	λ_{ex}	λ_{em}	%Q	λ_{ex}	λ_{em}	%Q
D, A									
Fl, TMR	495	520	94	495	580	69	552	580	75
TMR, RX	552	580	94	552	607	90	580	607	94
Fl, RX	495	520	41	495	607	45	580	607	91
D, D									
Fl, Fl	495	520	55						
TMR, TMR	552	580	90						
RX, RX	580	607	90						

For each measurement of a bichromophoric substrate that was made, a solution containing an equal concentration of the two respective singly labeled NorFES derivatives served as a control. Absorption, emission, and excitation spectra taken before and after addition of the protease were shown to be the same within experimental error as were spectra of the two cleaved solutions, i.e., the doubly labeled and equal concentrations of singly labeled peptides.

Since some of the spectroscopic data were unexpected, we applied a slight variation to the method of measuring enzymatic activity with heterobichromophoric substrates. Thus, using Fl-NorFES-RX, in addition to exciting into the nominal Förster donor, i.e., fluorescein, and observing its change in emission as a function of time after addition of enzyme, we also excited into the nominal acceptor, i.e., rhodamine-X, and followed this dye's fluorescence intensity at 607 nm. Data presented in Figure 5 show a greater than 2-fold enhanced signal sensitivity of RX over Fl.

Discussion

There is a large volume of literature in which the presence of two spectroscopically complementary fluorophores is used to determine distances within and between macromolecules.[4−8,12] The theory developed by Förster[1] is most frequently invoked to interpret these data. In cell-free systems probes such as dansyl and pyrene, which are believed to act as Förster acceptors for a naturally occurring donor, e.g., tryptophan in proteins, are commonly chosen; to overcome autofluorescence in whole cell systems, longer wavelength dyes such as the xanthenes, e.g., fluoresceins and rhodamines, are frequently employed as Förster donors and acceptors, respectively. Regardless of the probes used, the most common method of detection is to excite into the donor and observe the degree of quenching at the donor's emission peak.

An essential feature of Förster theory is spectral independence of the two dyes wherein the absorption spectrum of a bichromophoric species is the sum of its components' spectra.[13,14] Also according to this theory, if the emission of the donor is quenched by the acceptor, then the energy that would be radiatively emitted by the donor in the absence of the acceptor must be nonradiatively transferred in the latter's presence. Concomitantly, if the latter has a nonzero quantum yield, then the energy transferred to the acceptor must reappear as a radiative emission. Therefore, if only Förster type resonance energy transfer (FRET) is operative between two fluorophores, the emission of the acceptor must be higher in the presence of the donor than in its absence.

NorFES as a Vehicle for Examining Two Dipoles in Close Proximity. In a recent study, NorFES, an undecapeptide designed to serve as a substrate for the serine protease elastase, was labeled with either TMR or RX; absorption and fluorescence spectra of these two singly labeled peptides, i.e., TMR-NorFES and NorFES-RX, were then compared with those of NorFES heterodoubly labeled, i.e., TMR-NorFES-RX, to determine the number of photons absorbed and emitted by each fluorophore when simultaneously covalently bound to the same peptide backbone. It was shown that even though 90% of the fluorescence of TMR in TMR-NorFES-RX was quenched, not more than 4% of the photons absorbed by TMR, the nominal Förster donor, was transferred to RX, the nominal Förster acceptor.[11] Quenching of the intact peptide was attributed to the formation of a hetero dye ground-state dimer similar in structure to that proposed for TMR-NorFES-TMR.[9] In the latter the 34 nm blue-shifted peak of the intact peptide's absorption spectrum relative to that of the cleaved solution's was used to calculate a dipole−dipole separation (**R**) of ca. 6 Å using the Simpson−Peterson approximation where the magnitude of the specral shift (*U*/*hc*) is related to **R** by the following equation:

$$U = \frac{|d|^2}{n^2\mathbf{R}^3}|\kappa| \tag{3}$$

$|d|^2$ is the squared module of the transition dipole, n is the solvent refractive index ($n = 1.333$ in water), κ is the orientation factor,

$$\kappa = \cos\theta_{12} - 3\cos\theta_{1\mathbf{R}}\cos\theta_{2\mathbf{R}} \tag{4}$$

and θ_{12}, $\theta_{1\mathbf{R}}$ and $\theta_{2\mathbf{R}}$ represent the angles between the transition dipoles in dyes 1 and 2, between dipole 1 and the radius vector **R** connecting the two dipoles, and between dipole 2 and the radius vector **R**, respectively. Since for this homodoubly labeled molecule only a blue shift was observed along the 552 nm transition dipole axis (the N−N axis) and a small red shift for the dipole axis perpendicular to this plane (353 nm transition in the monomer), the following values were used in the calculation: $\theta_{12} = 0$ and $\theta_{1\mathbf{R}} = \theta_{2\mathbf{R}} = 90$. Thus, the proposed structure has two dyes coplanar in a side-by-side head to tail configuration. We envision a modified but similar structure for the heterodoubly labeled NorFES substrates (vide infra).

In the present work we compared the spectral properties of two additional heterodoubly labeled protease substrates before and after addition of the serine protease elastase (the latter resulted in separation of the two fluorophores to effectively infinite distance). In toto we used three heterocombinations of three xanthene dyes: fluorescein, tetramethylrhodamine, and rhodamine-X. In all three heterobichromophoric peptide solutions the fluorescence of the nominal Förster donor was decreased relative to the same solutions after cleavage of the substrates (Table 2). However, contrary to Förster theory, in all three peptides the fluorescence of the nominal acceptor was also quenched. In fact in the case of Fl-NorFES-RX, the acceptor's fluorescence, i.e., RX, was reduced to a greater extent than that of the donor's, i.e., Fl's, (91% vs 41%). In an earlier study transposition of the donor and acceptor was shown not to affect the spectral data.[9] Furthermore, the absence of emission spectral shape changes suggested the absence of exciplex formation. Therefore, an understanding of the mechanism responsible for the changes in fluorescence intensity in these substrates was sought.

Exciton Theory to Describe Strong Dipole−Dipole Interactions. In the spectra presented in Figures 1−3 the existence of dipole−dipole interactions is evident for all three of the heterobichromophoric substrates. None of the nine spectra could be arrived at by linearly combining spectra of the two fluorophore components present in any of the doubly labeled species. This is due to the loss of spectral independence of the individual dyes as a result of the intramolecular interaction between the two transition dipoles. Thus, one no longer sees spectra of the individual xanthenes but of a hybrid species with unique characteristics.

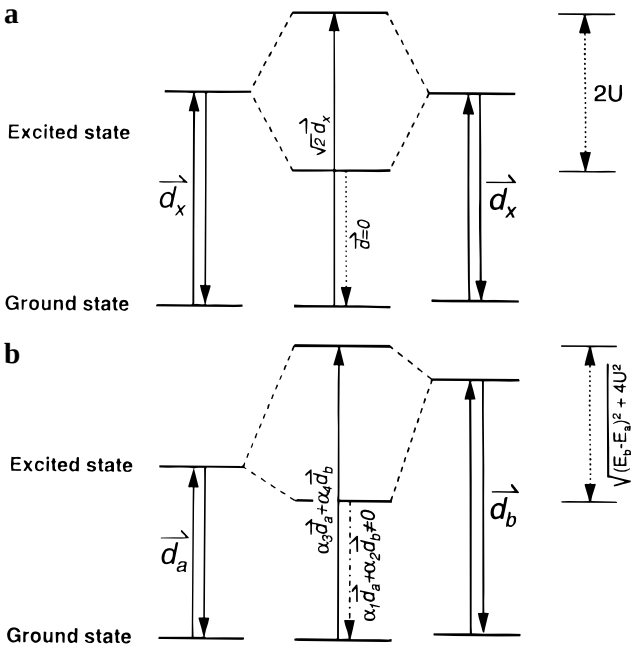

Figure 4. Exciton theory applied to dimer formation between two fluorophores. (a) Energy diagram for homodimers. The transition dipole between the ground and excited states of the monomers is set equal to d_x. Interaction between two identical dyes results in a splitting of the excited state into two states, each equally spaced from the monomer's excited state. For H-dimers, transitions between the ground and upper excited states are equivalent to $\sqrt{2}d_x$ and those between the ground and lower excited states are forbidden, i.e., the transition dipole equals zero (vice versa for J-dimers). The energy difference between the two split excited states is $2U$ where $U = (|d_x|/n^2R^2)|\kappa|$ and n is the refractive index of the medium (1.333 for water), R is the distance between centers of the point−dipole systems, and κ is the orientation factor as described in the text. (b) Energy diagram for heterodimers. The transition dipoles between the ground and excited states of the two monomers (a and b) are set equal to d_a and d_b, respectively. Interaction between the two different dyes also results in splitting of the excited state. Here, the transition dipole between the ground and new upper excited states is described by $a_3d_a + \alpha_4d_b$ and that between the ground and new lower excited states by $a_1d_a + \alpha_2d_b$. For H-dimers α_1 and α_2 have opposite signs and α_3 and α_4 the same sign (vice versa is true for J-dimers). The energy difference between the two split excited states is $((E_b - E_a)^2 + 4U^2)^{1/2}$ where $U = [(|d_a||d_b|)/n^2R^2]|\kappa|$.

Interaction between two identical dyes will result in excitation delocalization such that the spectral shape of the dimer formed by the two monomeric fluorophores will be markedly distorted from that of the monomer.[11] According to exciton theory,[15−18] the distortion results from the noninteracting dyes' doubly degenerate excited energy level splitting into two levels upon dye−dye interaction. In the case of two identical dyes (Figure 4a) the two new excited states will be separated by $2U$ where U is as defined in eq 3. In the case of H-dimers $\theta_{12} = 0°$, $\theta_{1\mathbf{R}} = \theta_{2\mathbf{R}} = 90°$, which yields $\kappa = 1$, whereas for J-dimers $\theta_{12} = \theta_{1\mathbf{R}} = \theta_{2\mathbf{R}} = 0°$, which yields $\kappa = -2$.

Spectroscopically, formation of H-homodimers results in a blue shift in the absorption spectrum and fluorescence quenching

whereas with J-homodimer formation a red shift and enhanced fluorescence are observed. The reasons for these spectral changes are shown in Figure 4a: for H-homodimers transitions between the ground state and upper excited state are permitted and those between the ground and lower excited state are forbidden whereas for J-homodimers the opposite is true. In Table 2 quenching of the three homodoubly labeled substrates is presented and is consistent with our previously provided spectral evidence for the existence of H-type dimers in bis-rhodamine NorFES derivatives as well as the model structure for TMR-NorFES-TMR.[9] The substantially lower degree of quenching for Fl-NorFES−Fl (55% vs 90% for each of the bisrhodamine derivatives) has been ascribed to the absence of charge on fluorescein's xanthene system.[10]

The transition dipole for the ground to lower excited state of a dimer is equal to $\alpha_1 d_a + \alpha_2 d_b$ and that of the ground to upper excited state $\alpha_3 d_a + \alpha_4 d_b$. H-dimers α_1 and α_2 have opposite signs and α_3 and α_4 the same sign; the reverse is true for J-dimers. Since for either type of homodimer the magnitudes of α_1 through α_4 are equal, in the case of H-homodimers the transition dipole between the ground and lower excited state is zero and that between the ground and upper excited state is $\sqrt{2}d_x$.

Figure 4b is presented in order to examine dipole−dipole interactions between two different dyes. In this case where d_a and d_b may not be equal in magnitude (Table 1), splitting of the two excited states will be by $2(U^2 + ((E_a - E_b)/2)^2)^{1/2}$ where

$$U = \frac{|d_a||d_b|}{n^2 R^3} |\kappa| \tag{5}$$

Additionally, the coefficients α_1 through α_4 are expected to all be different and, therefore, both blue- and red-shifted bands are observed, regardless of the geometry. However, in H-type geometry ($\theta_{12} = 0°$, $\theta_{1R} = \theta_{2R} = 90°$) the blue-shifted absorption band will be enhanced and the red-shifted decreased relative to a mixture of noninteracting dyes. Fluorescence, which is associated with transitions from the lowest excited state to the ground state, will be decreased, but not to zero, in the heterodimer.

The fluorescence observed at any particular pair of excitation and emission wavelengths is proportional to two probabilities: the probability of absorption of light at the excitation wavelength and the probability of radiative emission at the emission wavelength. For a spectroscopic species that is the product of individual fluorophores such as in the case of an H-dimer, these probabilities will be determined by the magnitudes and orientations of the transition dipoles of the unperturbed monomers and the relative magnitudes of U and $E_b - E_a$.[13] Consistent with this, the fluorescence of both TMR-NorFES-TMR and RX-NorFES-RX is quenched by 90% compared with only 55% for Fl-NorFES−Fl (Table 1).

In cases where the spectroscopic species is a product of two dyes, the energies of the delocalized excited states of the dimer will determine the wavelength maxima whereas the magnitudes of corresponding transition dipoles will determine the probabilities of transitions. Exciton bands in spectra resulting from heterodimers relative to a linear combination of individual fluorophore components' spectra will depend on the dipole−dipole interaction in the heterodimer. For example, of the three heterobichromophoric substrates, the largest changes in absorption and fluorescence spectra were observed in the TMR-NorFES-RX molecule since both a very prominent exciton band in the absorption spectrum (Figure 2a) and a large decrease in fluorescence at both fluorophores' emission maxima (Figures 2b,c) are observed. Next is Fl-NorFES-TMR where spectra indicate the fluorescence of Fl, the nominal donor, is quenched as would be expected of two independent fluorophores with good spectral overlap; however, quenching of the TMR (Figure 1c) and distortions in the absorption spectrum (Figure 1a) point toward existence of a dipole−dipole interaction of intermediate strength. Last, the smallest spectral changes are observed in Fl-NorFES-RX. In this substrate only the red side of the absorption spectrum is affected (Figure 3a) while fluorescence of the nominal acceptor (Figure 3c) is quenched by more than that of the nominal Förster donor (Figure 3b).

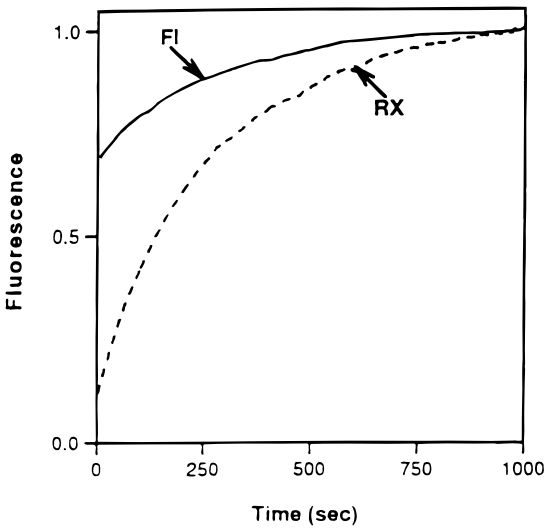

Figure 5. Fl-peptide-RX as a probe for measuring protease activity. The fluorescence intensity of Fl-NorFES-RX as a function of time after addition of the elastase is plotted by monitoring with the monochromators set at fluorescein's maxima ($\lambda_{ex} = 495$ nm and $\lambda_{em} = 520$ nm) (solid line) and at rhodamine-X's maxima ($\lambda_{ex} = 580$ nm and $\lambda_{em} = 607$ nm) (dashed line).

On the basis of the above results Fl-NorFES-RX was used as a probe of protease activity in two different ways: first, by exciting into the donor (Fl) and observing the fluorescence at 520 nm (Fl's emission peak) and then by observing both excitation and emission at the acceptor's (RX's) maxima, i.e., 580 and 607 nm, respectively. Figure 5 shows an increase in fluorescence intensity of 41% for Fl versus 91% for RX after addition of elastase to a solution of Fl-NorFES-RX. Thus, with this donor/acceptor pair, the signal sensitivity for the nominal acceptor is twice that for the nominal donor, thereby suggestive of an alternative type of spectroscopy. In the latter, called "Retsrof", fluorescence is monitored at the excitation and emission peaks of the more red-shifted fluorophore instead of those of the more blue-shifted fluorophore as is the case with classical Förster dye pairs. Since the absorption spectrum of Fl in Fl-NorFES-RX is not affected by the presence of RX, one has two distinct signals: the absorption of Fl which can serve as a measure of concentration and the dequenching of RX which can serve as a measure of proteolytic activity.

In the past it has been well-recognized that a number of difficulties exist in applying the Förster equation to some bichromophoric systems. In one of his last publications, Förster himself discussed some of these problems.[19] The lack of a universally accepted method for calculating κ^2 is such an example. A second case is the presence of exciton bands in absorption spectra; the latter indicates the existence of dipole−dipole interactions of strength greater than that for which Förster theory is applicable. In this paper we show that the absence of

major alterations in the absorption spectrum does not rule out the existence of dipole−dipole interactions of strength greater than that which can be described by the Förster model. Hence, although a comparison of the absorption spectra of the pre- and postcleaved Fl-NorFES-RX solutions in Figure 3a indicates only small changes and therefore a weak dipole−dipole interaction, the fluorescence spectra of the two solutions differ to a large extent, particularly at wavelengths associated with the acceptor (Figure 3c). Thus, quenching of the acceptor's fluorescence by the donor supports a mechanism which does not fit Förster theory. In a manuscript that is currently under review we address the influence of backbone length and flexibility on intramolecular H-dimer formation.[20]

Nomenclature

Fl, carboxyfluorescein
FRET, Förster-type resonance energy tranfer
Fmoc, N_α-9-fluorenylmethoxycarbonyl
PyBOP,benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
NMP, 1-methyl-pyrrolidinone
NMM, 4-methylmorpholine
HOBT, 1-hydroxybenzotriazole
DIEA, diisopropylethylamine
TFA, trifluoroacetic acid
TMR, carboxytetramethylrhodamine
HPLC, high-pressure liquid chromatography
NorFES, elastase substrate
RX, carboxyrhodamine-X
U, energy of dipole−dipole interaction
$\Delta\epsilon$, width of the absorption spectrum expressed in energy units

References and Notes

(1) Förster, T. *Ann. Phys*. **1948**, *2*, 55−75.
(2) Latt, S. A.; Cheung, H. T.; Blout, E. R. *J. Am. Chem. Soc*. **1965**, *87*, 995−1003.
(3) Stryer, L.; Haugland, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 719−726.
(4) Fairclough, H.; Cantor, C. R. *Methods Enzymol*. **1978**, *48*, 347−379.
(5) Wu., P.; Brand, L. *Anal. Biochem*. **1994**, *218*, 1−13.
(6) VanDer Meer, B. W.; Coker, G., III; Chem, S.-Y. S. *Resonance Energy Transfer, Theory and Data;* VCH: New York, 1994
(7) Selvin, P. *Methods Enzymol*. **1995**, *246*, 300−334.
(8) Clegg, R. M. *Curr. Opin. Biotech.* **1995**, *6*, 103−110.
(9) Packard, B. Z.; Toptygin, D. D.; Komoriya, A.; Brand, L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11640−11645.
(10) Packard, B. Z.; Komoriya, A.; Toptygin, D. D.; Brand, L. *J. Phys. Chem. B.* **1997**, *101*, 5070−5074.
(11) Packard, B. Z.; Toptygin, D. D.; Komoriya, A.; Brand, L. *Biophys. Chem*. **1997**, *67*, 167−176.
(12) Stryer, L. *Annu. Rev. Biochem.* **1978**, *47*, 819−846.
(13) Förster, Th.; König, E. *Ber. Bunsen-Ges. Phys.* **1957**, *61*, 344−348.
(14) Förster, Th. *Discuss. Faraday Soc*. **1959**, *27*, 7−17.
(15) Davydov, A. S. *Theory of Molecular Excitons*; McGraw-Hill: New York, 1962.
(16) Kasha, M. *Physical and Chemical Mechanisms in Molecular Radiation Biology*; Glass, W. A., Varma, M. N., Eds.; Plenum Press: New York, 1991; 231−255.
(17) Kasha, M.; Rawls, H. R.; Ashraf El-Bayoumi, M. *Pure Appl. Chem.* **1965**, *Vol. II*, 371−392.
(18) Kasha, M. *Rad. Res.* **1963**, *20*, 55−71.
(19) Förster, Th. *Modern Quantum Chemistry. Part III: Action of light and organic crystals;* Sinanoglu, O., Ed.; Academic Press: New York, 1965.
(20) Packard, B. Z.; Komoriya, A.; Nanda, V.; Brand, L. submitted to *J. Phys. Chem.*.