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Characterization of fluorescence quenching in bifluorophoric protease substrates

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Abstract

NorFES is a relatively rigid, bent undecapeptide which contains an amino acid sequence that is recognized by the serine protease elastase (AspAlaIleProNle ↓ SerIleProLysGlyTyr (↓ indicates the primary cleavage site)). Covalent attachment of a fluorophore on each side of NorFES's elastase cleavage site enables one to use a change of fluorescence intensity as a measure of enzymatic activity. In this study two bichromophoric NorFES derivatives, D-NorFES-A and D-NorFES-D, were prepared in which D (donor) was tetramethylrhodamine and A (acceptor) was rhodamine-X, two chromophores with characteristics suitable for energy transfer. Absorption and fluorescence spectra were obtained with both the intact and cleaved homodoubly, heterodoubly and singly labeled derivatives. It was found that both the homo and hetero doubly-labeled derivatives form ground-state complexes which exhibit exciton bands. The hetero labeled derivative exhibits little or no resonance energy transfer. Spectral measurements were also done in urea, which partially disrupts ground-state dimers. © 1997 Elsevier Science B.V.

Keywords: Fluorescence; Exciton bands; Förster-type resonance energy transfer; Ground-state complexes; Xanthenes; Protease substrates

1. Introduction

NorFES is an undecapeptide ((AspAlaIlePro-Nle ↓ SerIleProLysGlyTyr (↓ indicates the primary cleavage site)) which was designed and synthesized to serve as a substrate for the serine protease elastase [1]. Using the two amino groups located on opposite sides of the substrate's cleavage site and separated by seven amino acid residues, D-NorFES-D was made by covalently linking two tetramethylrhodamines (D) to the molecule. These chromophores form an intramolecular ground-state dimer with spectral characteristics that can be described by exciton theory. In contrast, Förster-type resonance energy transfer has previously been used to interpret data derived from other profluorescent protease substrates that contained two different dyes having spectral overlap between the donor emission and acceptor absorption [2–5]. If the Förster mechanism were operative the absorption spectra of the pre- and

Abbreviations: FRET, Förster-type resonance energy transfer; D, donor (tetramethylrhodamine); A, acceptor (rhodamine-X); D-NorFES-D, homodoubly-labeled elastase substrate; D-NorFES-A, heterodoubly-labeled elastase substrate; U, energy of dipole–dipole interaction; $\Delta\epsilon$, width of the absorption spectrum, expressed in energy units

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postcleaved peptides would be identical; additionally, the emission intensity of the putative acceptor fluorophore would be decreased and the emission spectrum of the solution would be blue shifted after addition of the protease. In order to assess the role of the acceptor in a putative Förster dye pair, we similarly synthesized D-NorFES-A in which tetramethylrhodamine (D) and rhodamine-X (A) were covalently attached to the same amino groups as in D-NorFES-D.

We now present a spectroscopic comparison of the NorFES polypeptide double labeled with both complementary dyes and identical fluorophores. Absorption and fluorescence spectra were also obtained in urea, which decreases ground-state complex formation.

2. Materials and methods

2.1. Peptide synthesis and derivatization

The reagents and methods used for peptide synthesis and derivatization have been described previously [1].

2.2. 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.

2.3. Spectroscopy

For all measurements the concentration of each peptide was 1 μM. Concentrations of substrates were determined by amino acid analyses.

2.4. Absorption spectra

All absorption measurements were made with a Shimadzu UV 160U spectrophotometer at room temperature with 1 cm pathlength cuvettes.

2.5. Fluorometry

All fluorescence measurements were carried out at 37°C with an SLM 48000 S spectrofluorometer (SLM–Aminco, Rochester, NY) using the same 1 cm cuvettes as in the absorption measurements. Excitation was with a Xenon arc lamp through a monochromator and a polarizer 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.

3. Results

The undecapeptide NorFES (DAIPN$_l$SIPKGY) (N$_l$ = norleucine) was designed and synthesized to serve as a substrate for the serine protease elastase. Using the two amino functionalities of this molecule, i.e., the α-amino of the aspartic acid and the ϵ-amino of the lysine, D-NorFES-D was made by covalently linking tetramethylrhodamines on opposite sides of the peptide's cleavage site, and D-NorFES-A by covalently linking tetramethylrhodamine on one side and rhodamine-X on the other. The overall objective of this study was to compare the spectral properties of the two NorFES substrates. To this end we have measured absorption and fluorescence spectra of both doubly labeled peptides before and after cleavage. Since the data from early experiments in which D-NorFES-A was a substrate were not fully in accord with the Förster model of resonance energy

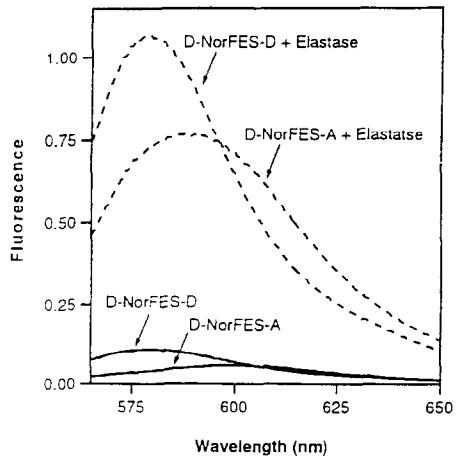

Fig. 1. Emission spectra of D-NorFES-D and D-NorFES-A before (solid line) and after (dashed line) addition of elastase. The excitation monochromator was set at 552 nm.

transfer [6], experiments were directed at defining the mechanism by which light is absorbed and excitation energy is lost by this substrate as well as by D-NorFES-D. Spectroscopic measurements were also done with both intact doubly-labeled and singly-labeled peptides in the presence and absence of the chaotropic agent urea.

The first set of experiments was carried out to compare the spectral properties of the two doubly-labeled substrates. Fig. 1 shows the fluorescence emission spectra of both doubly-labeled substrates before and after cleavage. Excitation was at 552 nm, the maximum of the donor excitation; the acceptor also exhibits some absorption at this wavelength. Although enhancements in the fluorescence intensity of solutions containing D-NorFES-A and D-NorFES-D are similar upon cleavage, 17- and 10-fold, respectively, the emission spectrum of cleaved D-NorFES-A exhibits a 17 nm blue shift relative to that of the intact molecule whereas only 2 nm separate the emission peaks in the pre- and postcleaved D-NorFES-D spectra. It appeared possible that the red-

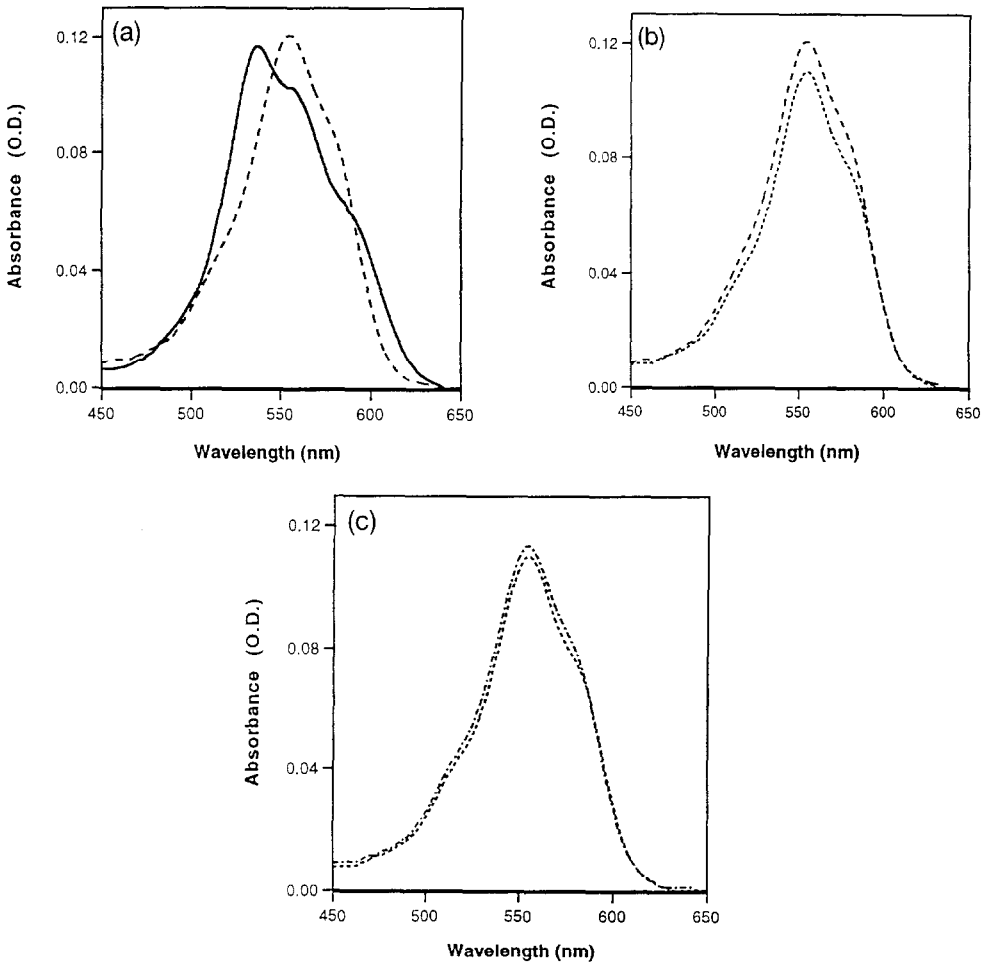

Fig. 2. Absorption spectra of (a) D-NorFES-A before (solid line) and after (dashed line) addition of elastase, (b) D-NorFES-A after addition of elastase (dashed line) and a solution containing an equimolar (1 μM) concentration of D-NorFES and NorFES-A (dotted line), and (c) a solution containing an equimolar (1 μM) concentration of D-NorFES and NorFES-A before (dotted line) and after (dotted–dashed line) cleavage by elastase.

shifted emission of the intact D-NorFES-A, as compared to D-NorFES-D, could be due to energy transfer and, therefore, this possibility was explored in more detail.

The absorption spectra of D-NorFES-A before and after cleavage are shown in Fig. 2a. They indicate that although the intact molecule contains two different dyes, it forms a ground-state complex with spectral properties that can be described by exciton theory. (Exciton theory was initially derived for and is most often applied to describe aggregates of identical monomers.) The 20 nm red shift in the absorption spectrum upon cleavage by elastase is consistent with a strong dye–dye interaction indicative of ground-state dimer formation in the precleaved substrate [7–17]. The Förster energy transfer model is inconsistent with changes in absorption spectra. As is shown in Fig. 2b, the absorption spectra of the cleaved peptide and an equimolar mixture of D-NorFES and NorFES-A are almost identical and quite different from the spectrum of intact D-NorFES-A. For further comparison, absorption spectra of this equimolar mixture both before and after elastase addition are shown in Fig. 2c.

Thus, since some aspects of the D-NorFES-A data were inconsistent with a Förster-type mechanism, two series of fluorescence spectra of this heterolabeled compound were compared with those of the solution containing equal concentrations of the singly-labeled peptides, i.e. D-NorFES and NorFES-A. These included three excitation spectra (Fig. 3) with emission set at 580, 607 and 670 nm and three emission spectra (Fig. 4) with excitation at 530, 552 and 580 nm. These data were used to separate the spectral contributions due to photons absorbed by D and emitted by D, absorbed by D and emitted by A, and absorbed by A and emitted by A.

3.1. Data analysis

In the approximation of the Förster model the energy of resonance dipole–dipole interaction between transition dipoles of the donor and acceptor is assumed to be small enough that it does not perturb the spectroscopic characteristics of the individual dyes such as extinction coefficients, radiative and nonradiative decay rates as well as shapes of absorption and emission spectra. In this approximation the fluorescence spectrum can be represented as follows:

$$\begin{aligned} I(\lambda_{ex}, \lambda_{em}) &= S_{ex}(\lambda_{ex}) \times S_{em}(\lambda_{em}) \\ &\times \left[C_D \epsilon_D(\lambda_{ex}) \frac{k_{Dr}}{k_{Dr} + k_{Dnr} + k_{ET}} f_D(\lambda_{em}) \right. \\ &+ C_D \epsilon_D(\lambda_{ex}) \frac{k_{ET}}{k_{Dr} + k_{Dnr} + k_{ET}} \\ &\times \frac{k_{Ar}}{k_{Ar} + k_{Anr}} f_A(\lambda_{em}) + C_A \epsilon_A(\lambda_{ex}) \\ &\left. \times \frac{k_{Ar}}{k_{Ar} + k_{Anr}} f_A(\lambda_{em}) \right] \end{aligned} \quad (1)$$

where $I(\lambda_{ex}, \lambda_{em})$ is the fluorescence intensity reading at the excitation wavelength λ_{ex} and the emission wavelength λ_{em}, $S_{ex}(\lambda_{ex})$ and $S_{em}(\lambda_{em})$ are the functions describing variations of instrument sensitivity with the excitation and emission wavelengths, C_D and C_A are the molar concentrations of the donor and acceptor, $\epsilon_D(\lambda_{ex})$ and $\epsilon_A(\lambda_{ex})$ are the extinction coefficients of the two fluorophores, $f_D(\lambda_{em})$ and $f_A(\lambda_{em})$ are the absolute shapes of the emission spectra (integrals of these functions over their entire emission spectra equal unity), k_{Dr} and k_{Ar} are the radiative decay rates, k_{Dnr} and k_{Anr} are the nonradiative decay rates, and k_{ET} is the Förster energy transfer rate.

Eq. (1) can be simplified by introducing of the functions $E_n(\lambda_{ex})$ and $F_n(\lambda_{em})$ $(n = 1, 2, 3)$ defined as follows:

$$E_1(\lambda_{ex}) = E_2(\lambda_{ex}) = S_{ex}(\lambda_{ex}) \epsilon_D(\lambda_{ex}) \quad (2)$$

$$E_3(\lambda_{ex}) = S_{ex}(\lambda_{ex}) \epsilon_A(\lambda_{ex}) \quad (3)$$

$$F_1(\lambda_{em}) = S_{em}(\lambda_{em}) \frac{k_{Dr}}{k_{Dr} + k_{Dnr}} f_D(\lambda_{em}) \quad (4)$$

$$F_2(\lambda_{em}) = F_3(\lambda_{em}) = S_{em}(\lambda_{em}) \frac{k_{Ar}}{k_{Ar} + k_{Anr}} f_A(\lambda_{em}) \quad (5)$$

Eq. (1), rewritten in terms of the functions defined above, becomes:

$$I(\lambda_{ex}, \lambda_{em}) = \sum_{n=1}^{3} \alpha_n E_n(\lambda_{ex}) F_n(\lambda_{em}) \quad (6)$$

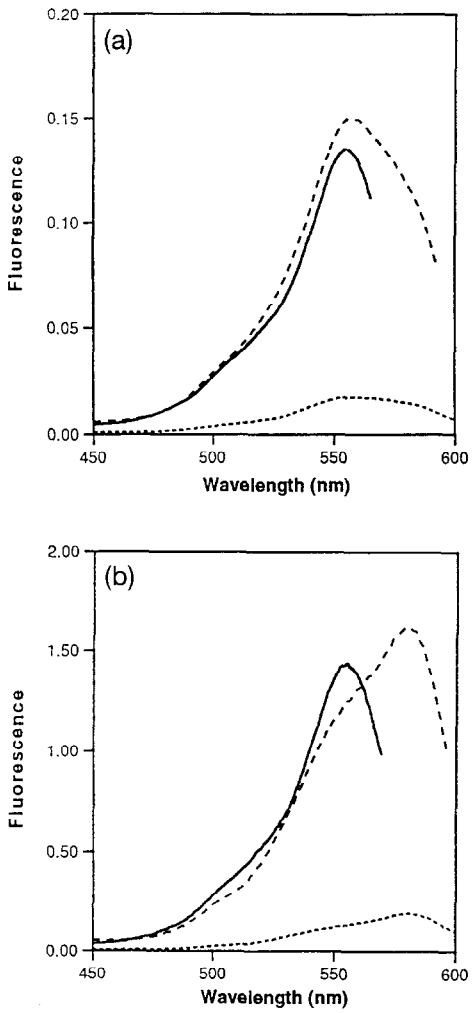

Fig. 3. Excitation spectra with the emission set at 580 nm (solid line), 607 nm (dashed line) and 670 nm (dotted line) for (a) D-NorFES-A and (b) an equimolar mixture of D-NorFES plus NorFES-A.

The values of the coefficients α_1, α_2 and α_3 can be expressed by substituting the functions defined in Eqs. (2–5) into Eq. (6) and matching the result with Eq. (1), which yields:

$$\alpha_1 = (1 - \phi_{ET})C_D \tag{7}$$

$$\alpha_2 = \phi_{ET}C_D \tag{8}$$

$$\alpha_3 = C_A \tag{9}$$

where the quantity ϕ_{ET} represents the quantum efficiency of the Förster energy transfer,

$$\phi_{ET} = \frac{k_{ET}}{k_{Dr} + k_{Dnr} + k_{ET}} \tag{10}$$

If the Förster model of energy transfer is applicable, then the following relations between the coefficients α_1, α_2 and α_3 should hold:

$$\alpha_1 + \alpha_2 = C_D,\ \alpha_3 = C_A \tag{11}$$

In contrast, violation of relation (11) is indicative of a mechanism other than Förster energy transfer. In

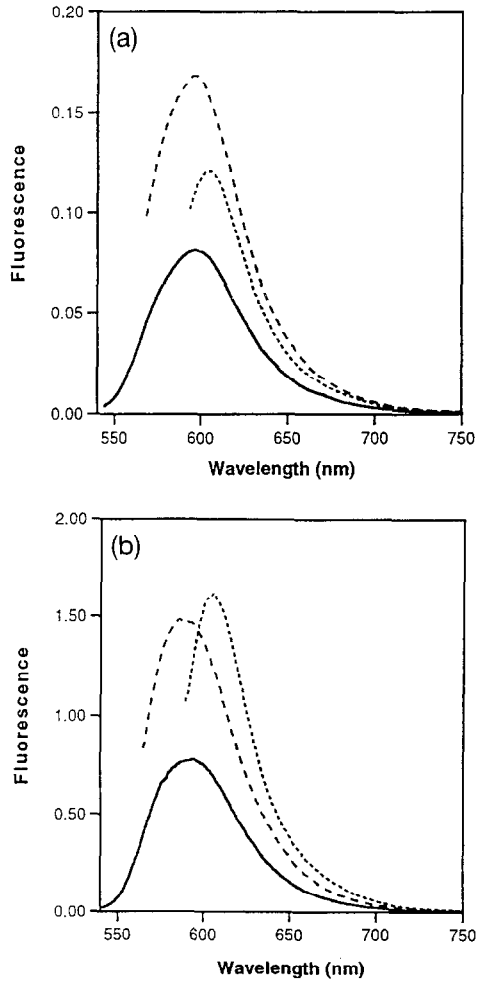

Fig. 4. Emission spectra with the excitation set at 530 nm (solid line), 552 nm (dashed line) and 580 nm (dotted line) for (a) D-NorFES-A and (b) an equimolar mixture of D-NorFES plus NorFES-A.

this connection it is important to know whether Eq. (6) can be resolved with respect to the unknowns α_1, α_2 and α_3 and whether the solution is unique. In order to address this question the values of the products $E_n(\lambda_{ex}) \cdot F_n(\lambda_{em})$ on a set of pairs of arguments $(\lambda_{ex}, \lambda_{em})$ must be considered as a vector. If the three vectors corresponding to $n = 1$, 2, and 3 are linearly independent, then there is a solution and it is unique. The solution can be obtained by linear regression analysis. If the three vectors are linearly dependent, then a unique determination of α_1, α_2 and α_3 is not possible. The latter is the case when: (i) the excitation wavelength is not varied in the experiment; (ii) the emission wavelength is not varied in the experiment; (iii) the energy acceptor has zero quantum yield; or (iv) the donor and acceptor are identical molecules and therefore their spectra are indistinguishable. If none of these four conditions is satisfied, then a unique determination of α_1, α_2 and α_3 is possible.

In practice, the data analysis was performed in two steps. In the first step the functions $E_n(\lambda_{ex})$ and $F_n(\lambda_{em})$ were determined from the spectroscopic data obtained with solutions of donor alone (D-NorFES) and acceptor alone (NorFES-A), i.e., singly-labeled peptides alone. In each case the data included a family of excitation spectra (using different emission wavelengths), a family of emission spectra (using different excitation wavelengths), and an absorption spectrum. The program that accomplished the first step is essentially a nonlinear least-square algorithm. The reduced χ^2 calculated by this program can be used to distinguish a spectroscopically homogeneous dye solution ($\chi^2 = 1$) from a mixture of several spectroscopic species ($\chi^2 \gg 1$).

Table 1

Peptide	C_D [a]	C_A [a]	α_1 [a,b]	α_2 [a,b]	α_3 [a,b]
D-NorFES	1.00	0.00	1.00	0.00	0.00
NorFES-A	0.00	1.00	0.00	0.00	1.00
D-NorFES + NorFES-A	1.00	1.00	1.17	0.02	1.10
D-NorFES-A	1.00	1.00	0.10	0.04	0.07

[a] The values of the concentrations C_D, C_A and coefficients α_1, α_2, α_3 are expressed in μM^{-1}.

[b] The coefficient α_1 represents the contribution from the quanta absorbed and emitted by the donor, α_2 represents the contribution from the photons absorbed by the donor and emitted by the acceptor, and α_3 represents the contribution from the photons absorbed and emitted by the acceptor. The values of these coefficients must satisfy Eq. (11) in the text if the Förster theory is obeyed.

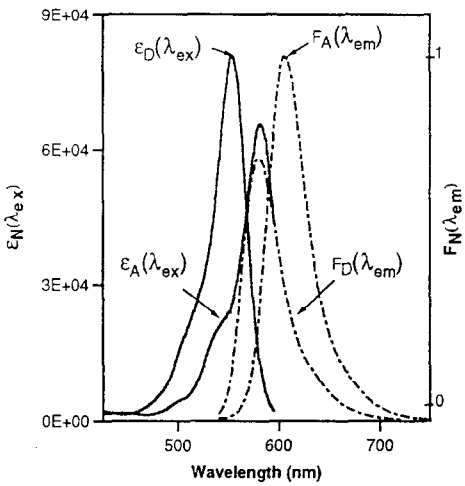

Fig. 5. The functions $E(\lambda_{em})$ (solid lines) and $F(\lambda_{em})$ (dashed–dotted line) for tetramethylrhodamine (D) and rhodamine-X (A) covalently bound to NorFES. Functions are defined in Eq. (6) where $E_A = E_1 = E_2$, $E_D = E_3$, $F_A = F_1$, and $F_D = F_2 = F_3$.

In the second step the data obtained with any combination of donor and acceptor were analyzed in order to obtain α_1, α_2 and α_3. The program that accomplished the second step is essentially a linear least-square algorithm that also calculates the reduced χ^2. The results of such an analysis are shown in Table 1, in which the value of each alpha represents the contribution of each of these three components to the linear combination given by Eq. (6). Thus, when only the donor is present $\alpha_1 = C_D$ and $\alpha_2 = \alpha_3 = 0$; when only the acceptor is present $\alpha_1 = \alpha_2 = 0$ and $\alpha_3 = C_A$. If 100% efficient energy transfer takes place, then $\alpha_1 = 0$, $\alpha_2 = C_D$ and $\alpha_3 = C_A$.

The values of α_1, α_2 and α_3 obtained for D-NorFES, NorFES-A and the mixture of D-NorFES and NorFES-A match expectations within experimental error. In contrast, for D-NorFES-A the sum $\alpha_1 + \alpha_2$ is less than C_D and α_3 is less than C_A (Eq. (11)). The former indicates that the excitation quanta that disappear from the donor do not reappear in the acceptor, and the latter indicates that the emission of the acceptor is significantly quenched.

Fig. 5 shows plots of spectral functions $E(\lambda_{ex})$

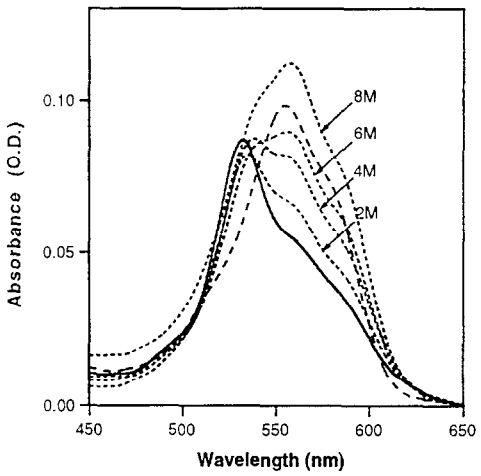

Fig. 6. Absorption spectra of D-NorFES-A (solid line), in the presence of 2 M, 4 M, 6 M and 8 M urea (dotted lines) and after addition of 1 U elastase (dashed line).

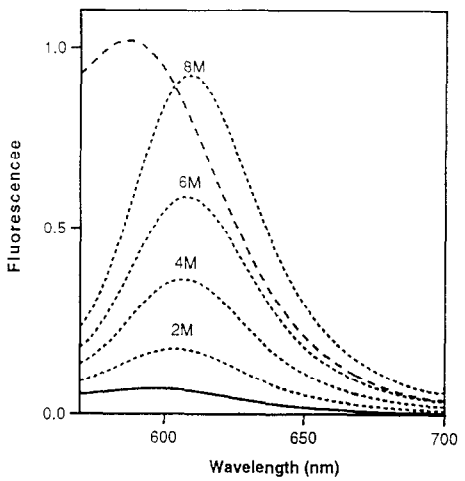

Fig. 8. Emission spectra of D-NorFES-A (solid line), in the presence of 2 M, 4 M, 6 M and 8 M urea (dotted lines) and after addition of 1 U elastase (dashed line). The excitation monochromator was set at 552 nm.

and $F(\lambda_{em})$ as defined in Eq. (2)–(5) for D and A. Since the area under the function $F_A(\lambda_{em})$ exceeds that under $F_D(\lambda_{em})$, the quantum yield of the acceptor is higher than that of the donor. Therefore, the loss of intensity at the donor's peak must be accompanied by a gain in intensity at the acceptor's peak if the Förster mechanism is in effect. Since this is not observed, an alternative mechanism must be in operation.

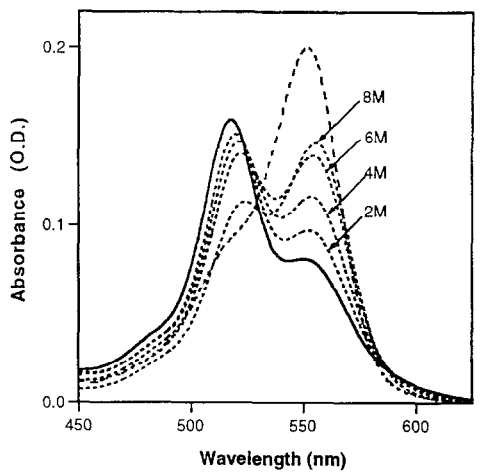

Fig. 7. Absorption spectra of D-NorFES-D (solid line), in the presence of 2 M, 4 M, 6 M and 8 M urea (dotted lines) and after addition of 1 U elastase (dashed line).

3.1.1. Effect of urea

Experiments were next directed at observing the effects of a chaotropic agent on the intramolecular dimerization. The absorption spectra are plotted in Fig. 6 for D-NorFES-A and in Fig. 7 for D-NorFES-D. Spectra of the peptides in the presence of urea ranging in concentration from 2 to 8 M are represented by the dotted lines. It can be seen that, as the urea concentration is raised, the blue-shifted peaks, at 536 nm for D-NorFES-A and at 518 nm for D-NorFES-D, which are characteristic of the intramolecular dimers, decrease and those at 556 and 552 nm, respectively, characteristic of the monomers, increase. These results are consistent with a urea-induced dye–dye separation. Urea had a small effect on the absorption spectra of singly-labeled NorFES peptides and it may also influence the spectra of doubly-labeled peptides to a similar extent. Addition-

Table 2

Peptide		Cleaved	0 M	2 M	4 M	6 M	8 M
D-NorFES-A	$\%I_{580}$	1.00	0.06	0.10	0.16	0.23	0.30
	$\%I_{607}$	1.00	0.07	0.21	0.47	0.77	1.20
	λ_{max}	584	601	604	604	607	608
D-NorFES-D	$\%I_{580}$	1.00	0.10	0.26	0.45	0.77	0.96
	λ_{max}	581	579	583	582	583	584

ally, in accord with these findings are the loss of quenching (Fig. 8), as are wavelength data, given in Table 2, in which the quenching of both peptides is shown to decrease and the emission maximum of D-NorFES-A to increase upon addition of urea.

4. Discussion

Chromogenic peptides have been used as substrates for the detection of protease activities for many years [5,18]. One type of chromogenic substrate is a peptide containing a cleavage site for the protease under study with a chromophore covalently bound on each side of this sequence. One such bichromophoric approach uses dyes that form a good resonance energy transfer pair where the emission spectrum of the first dye, the donor, overlaps with the excitation spectrum of the second, the acceptor. Quenching of the donor has been ascribed to the presence of the acceptor. Many workers [2–5] have utilized Förster-type resonance energy transfer to describe this quenching and sensitization of fluorescence.

Förster's theory was derived on the assumption that the dipole–dipole interaction is very weak [19]. If only the Förster mechanism were operative, then one would expect to see a decrease in the donor's emission intensity and an increase in the acceptor's. The Förster mechanism does not predict quenching of the acceptor by the donor as is observed in this study for D-NorFES-A.

4.1. Alternatives to the Förster theory

Examination of the fluorescence spectra of D-NorFES-A and comparison with those of D-NorFES and NorFES-A provide strong qualitative evidence for the involvement of mechanisms other than Förster-type resonance energy transfer (FRET) in this system. For example, a comparison of the fluorescence emission spectra of pre- and postcleaved heterodoubly-labeled peptide-containing solutions indicates that the emission of the precleaved peptide does not exceed that of the postcleaved at any wavelength (Fig. 1). This result is not consistent with FRET, where the emission of the precleaved peptide solution must be higher than that of the postcleaved on the red side of the spectrum.

As for the absorption spectroscopy of the heterodoubly-labeled peptide, the solid line in Fig. 2a which represents the intact peptide in buffer shows a maximum at 536 nm. This peak is 20 nm blue-shifted from that of a solution containing equal concentrations of the two singly-labeled NorFES peptides (dotted line in Fig. 2b) or as is observed after complete cleavage of D-NorFES-A (Fig. 2a and 2b). The shift is indicative of a ground-state dimer and the blue-shifted peak represents an exciton band.

4.2. Decomposition of spectral components

It was of interest to separate the spectral contributions due to the photons absorbed and emitted by the donor, absorbed by the donor and emitted by the acceptor, and those both absorbed and emitted by the acceptor. This separation was performed using the component excitation (Fig. 3) and emission (Fig. 4) spectra obtained with solutions of D-NorFES and NorFES-A. The wavelength-dependent extinction coefficients and relative emission spectra are shown in Fig. 5. Data in Table 1, in which results from the decomposition of spectra obtained with D-NorFES-A, a mixture of equal concentrations of D-NorFES and NorFES-A, and solutions containing the singly-labeled peptides alone are presented, indicate that in D-NorFES-A 90% ($[(C_D - \alpha_1)/C_D] \times 100\%$) of quanta absorbed by D disappear from D and not more than 4% ($[\alpha_2/C_D] \times 100\%$) reappear in A. This is a major difference from the result expected for a substrate if the Förster mechanism were operative.

4.3. Urea as a tool for intramolecular dye separation

In order to better understand the absorption and fluorescence of NorFES peptides the effects of urea on their spectral properties were determined. This chaotropic agent is expected to induce an extended conformation of the peptide backbone and to disrupt intramolecular dimers. The dotted lines in Figs. 6 and 7, which represent absorption spectra of the doubly-labeled peptides in 2, 4, 6 and 8 M urea, point toward incremental loss of the exciton bands.

At 8 M urea the peaks at 556 and 552 nm peaks, respectively, represent the sum of the monomers. These data are consistent with a urea-induced disruption of intramolecular dimers. The absence of an isosbestic point indicates the existence of more than two species, such as a single dye–dye dimer and free monomers. Thus, results obtained for the intact peptides are those expected for an ensemble of conformations for urea-induced extended peptide backbone structures.

Fluorescence data are also in accord with these interpretations (Table 2 and Fig. 8) as they too suggest a dye–dye separation. First, as the urea concentration is increased from 0 to 8 M, the intensity at 607 nm, the acceptor's peak, increases from 7 to 120% of the intensity of a solution containing the fully cleaved peptide. Second, the emission wavelength maximum increases from 601 to 608 nm. Thus, the implication is of a decrease in the strength of the resonance dipole–dipole coupling, or a decrease in the dimer/monomer ratio.

4.4. Ground state dimers with exciton absorption bands and quenched fluorescence

The data shown in this paper indicate that the quenching of fluorescence observed in D-NorFES-A as well as in D-NorFES-D can be explained in terms of ground-state dimer formation with exciton absorption bands [20]. This is reflected in the blue shifts in the absorption spectra of the intact peptides (Fig. 2a, 6 and 7). For D-NorFES-D the magnitude of the exciton splitting ($2U$) is 2400 cm^{-1} and that of the Franck–Condon bandwidth ($\Delta\epsilon$) is 1500 cm^{-1}. As the concentration of urea is increased, the percent dimer decreases. However, since a blue shoulder never disappears from the absorption of intact D-NorFES-A, even in the presence of 8 M urea where the intensity at 607 nm, the acceptor's peak, actually exceeds that of the cleaved solution, the very weak coupling between fluorophores as described by Förster is not present for the entire ensemble of conformations of D-NorFES-A under any conditions described here. Rather, the data in the presence of urea support a dipole–dipole coupling intermediate in strength between the strong coupling that results in exciton bands and the Förster model for resonance energy transfer.

The data presented in this paper illustrate several important features regarding the strength of resonance dipole–dipole interactions between two fluorophores. Our conclusion that this interaction can be decreased from the tight coupling of the intact peptides in buffer alone (in which clear exciton bands can be observed) to the 8 M urea data, where less pronounced but finite spectral shape changes are present, should give caution to the interpretation of spectroscopic data. Specifically, use of the Förster model should be invoked only where absorption spectra of the quenched and free fluorophores are identical and the increase in emission intensity of the acceptor is quantifiable. Otherwise, coupling between fluorophores may be indicative of a mechanism different from that described by Förster.

Application of the principle of strong dipole–dipole interactions to the design of protease substrates as well as to the conformational analysis of macromolecules could provide insights into biologically significant structures and functions. In this regard, by comparing the spectral properties of NorFES homodoubly-labeled with six different fluorophores, we have recently found that the intramolecular dimerization described in this paper is not unique to rhodamines but can be observed with other fluorophores which fit a structural profile [21].

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