Intramolecular Excitonic Dimers in Protease Substrates: Modification of the Backbone Moiety To Probe the H-Dimer Structure†

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Received: October 22, 1997; In Final Form: January 5, 1998

NorFES (DAIPN$_1$SIPKGY, N$_1$ = norleucine) is an undecapeptide that contains a recognition sequence and cleavage site for the serine protease elastase. When NorFES is doubly labeled with a variety of fluorophores on opposite sides of this amino acid sequence, the fluorescence is quenched due to formation of intramolecular ground-state dimers. Although the spectral characteristics of these dimers are predictable by exciton theory, influence of the peptide backbone on H-dimer formation is less well understood. Specifically, factors that modify the attractive forces between and orientation of dyes are not well-characterized. Thus, by varying the dye linker moieties, we have sought to evaluate the thermodynamic parameters for intramolecular H-type dye−dye association and the structures of these dimers. We now present data from a series of homodoubly labeled NorFES derivatives that differ by the addition of one or two 6-aminohexanoic acids to the peptide backbone. By comparing absorption and fluorescence properties of these substrates as a function of temperature, we examined how such additions could modify dimerization; we calculated the free energy of activation ($\Delta G^{\ddagger}$) for intramolecular dimer disruption of each substrate. To gain further insight into dye−dye orientation, a NorFES substrate modified to facilitate intramolecular H-dimerization was synthesized with different geometric dye isomers. The data show that length and conformation of the peptide plus linker as well as stereochemistry of dye−peptide conjugation play important roles in intramolecular ground-state complexation. The factors that influence the spectral properties of intramolecular H-dimerization support our earlier proposed model for H-dimers in NorFES peptides.

Introduction

We have recently shown that principles of exciton theory can be applied toward the design of profluorescent protease substrates.[1,2] Moreover, the protease-induced fluorescence enhancement in polypeptides doubly labeled with fluorophores can be used as a quantitative measure of proteolytic activity. Quenching, which occurs when two fluorophores of significant transition dipole strength, symmetry, and delocalized charge come within ca. 15 Å of each other, is believed to result from the formation of intramolecular ground-state H-dimers.[3−6] More specifically, we have previously characterized the spectral properties of a series of profluorescent substrates, each containing the undecapeptide named NorFES;[7−9] the latter includes a recognition amino acid sequence and cleavage site for the serine protease elastase. In those studies we examined possible mechanisms for observed absorption spectral changes and fluorescence quenching by comparing spectral properties of doubly and singly labeled NorFES derivatives before and after cleavage by elastase.

In the current study we examined the influence of conformational flexibility of the peptide linker moiety on the Gibbs free energy of intramolecular dye−dye association. We synthesized a series of NorFES substrates with 6-aminohexanoic acid additions at either the amino or carboxyl or both ends of the backbone linker moiety. Since the magnitude of dye−dye dimer formation cannot exceed the activation free energy for dimer dirsuption ($\Delta G^{\ddagger}$), using van't Hoff plot analyses of fluorescence data we determined the $\Delta G^{\ddagger}$ for each NorFES substrate. Furthermore, to derive a more detailed structure of rhodamine H-type intramolecular dimers, a comparison of absorption and fluorescence spectral properties of substrates with two distinct dye-linker stereochemistries was made.

Materials and Methods

Materials. Na-9-fluorenylmethoxycarbonyl (Fmoc) amino acids, Fmoc-amino acid resins (NovaSyn-TGA), and coupling reagent (PyBOP) were purchased from Calbiochem-Novabiochem (La Jolla, CA). Solvents such as HPLC-grade dichloromethane (DCM), methanol, and acetonitrile were from Fisher Scientific Co. (Pittsburgh, PA). Other reagents such as 1-methylpyrrolidinone (NMP), 4-methylmorpholine (NMM), 1-hydroxybenzotriazole (HOBT), diisopropylethylamine (DIEA), and protein-sequencing-grade trifluoroacetic acid (TFA) were from Aldrich Chemical Co. (Milwaukee, WI). Fluorophores 9-(2,5-dicarboxyphenyl)-3,6-bis(dimethylamino)xanthylium chloride (5-TMR), succinimidyl ester, 9-(2,6-dicarboxyphenyl)-3,6-bis-(dimethylamino)xanthylium chloride (6-TMR), 9-(2,5-dicarboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthene (5-DER),

† Abbreviations: DER, 9-(2-carboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthylium; 5-DER, 9-(2,5-dicarboxyphenyl)-2,7-dimethyl-3,6-bis-(ethylamino)xanthylium; 6-DER, 9-(2,6-dicarboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthylium; hex, 6-aminohexanoic acid; KNorFES, KDAIPN$_l$-SIPKGY; N$_l$, norleucine; NorFES, DAIPN$_l$SIPKGY; TMR, 9-(2-carboxyphenyl)-3,6-bis(dimethylamino)xanthylium; 5-TMR, 9-(2,5-dicarboxyphenyl)-3,6-bis(dimethylamino)xanthylium; $\Delta G^{\ddagger}$, free energy of activation for disruption of intramolecular H-type excitonic dimers.

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S1089-5647(97)03419-6 CCC: $15.00
Published on Web 02/14/1998

9-(2,6-dicarboxyphenyl)-2,7-dimethyl-3,6-bis(ethylamino)xanthene (6-DER), succinimidyl ester, and 6-(5-TMR)-carboxamidohexanoic acid, succinimidyl ester were from Molecular Probes (Eugene, OR), and porcine pancreatic elastase was from Sigma Chemical Co. (St. Louis, MO). C_{18} and C_4 reverse-phase high-pressure liquid chromatography (HPLC) columns were from Vydac, Inc. (Hesperia, CA) and SynChrom, Inc. (Lafayette, IN), respectively.

Methods. *Peptide Synthesis.* The reagents and methods used for peptide synthesis and derivatization have been described in detail previously.[1] Briefly, peptides (NorFES amino acid sequence is DAIPN$_l$SIPKGY, where the elastase cleavage site is between N_l (norleucine) and S) were synthesized using manual F-moc chemistry and were sequentially derivatized with the succinimidyl ester of each fluorophore. Crude as well as conjugated peptides were purified by reverse-phase HPLC including separation of two fluorophore isomers, when appropriate, 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 or 50 mM phosphate, pH 9 at 37 °C.

Spectroscopy. (a) Absorption Spectra. All absorption measurements were made with a Shimadzu UV 160U spectrophotometer equipped with a constant-temperature cuvette holder at the indicated temperature.

(b) Fluorometry. All fluorescence measurements were carried out 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° (the magic angle) to the vertical. Emission was observed through a monochromator and a vertical polarizer. Both monochromators had a resolution of 4-nm full-width at half-maximum.

Computer Simulations. All simulations were run in the CVFF force field using Discover 3.0 on a Silicon Graphics Indigo[2] workstation. The starting structures for molecular dynamics simulations consisted of the peptide bonds and linker arms in extended conformations. A simulated annealing protocol[10] was used to explore the conformational space and to extract end-to-end distance distributions. Specifically, each starting structure was minimized for 300 steps by the steepest descent method to a convergence of 1000 kcal/mol followed by a conjugate gradient method to 10 kcal/mol and finally a Newton minimization method to 0.001 kcal/mol convergence. Then the structure underwent a 100-ps dynamics simulation (time step of 1 fs) at 1000 K. (100 ps was determined to be sufficient for the structural distribution to reach equilibrium.) This was followed by a series of 1-ps dynamic simulations from 1000 to 400 K in 100 K decrements. The final structure was minimized for 300 steps using the same convergence limits as before. For each system simulated, 200 independent dynamic simulations were carried out. From these derived structures, the conformational energies as well as end-to-end distances between the terminal amide nitrogens where a dye would be conjugated to the backbone or a linker arm were determined.

Results

The aim of this work was to derive further insight into the intramolecular H-type excitonic dimer structure by evaluating the effect of temperature on the spectral properties of a series of protease substrates homodoubly labeled with rhodamines. To this end the distance between two rhodamines on opposite sides of the cleavage site of NorFES, an undecapeptide that was designed and synthesized to serve as a substrate for the serine protease elastase, was extended by synthesizing three NorFES−derived peptides, doubly labeling each with TMR, and comparing their spectral properties with those of the parent NorFES doubly labeled with TMR. The three synthesized derivatives were as follows: (1) NorFES with a 6-aminohexanoic acid covalently bound to the ϵ-amino group of lysine, the P_4' residue (NorFES−hex), (2) NorFES with a 6-aminohexanoic acid covalently bound to the α-amino group of the N-terminal aspartic acid, the P_5 residue (hex−NorFES), and (3) NorFES with two 6-aminohexanoic acids covalently bound, one to the ϵ-amino group of the P_4' residue and the other to the α-amino group of the P_5 residue (hex−NorFES−hex). We evaluated the influence of the different peptide-to-dye linker moieties by determining the free energy of activation ($\Delta G^{\ddagger}$) for disruption of the intramolecular H-dimers. On the basis of the analysis of the spectral data of these peptides, the NorFES backbone was then further modified by addition of a lysine to the amino terminus; this substrate, designated as KNorFES, was then homodoubly labeled with TMR and DER so that properties of the homodimers formed by these two similar but significantly different dyes could be compared. The dye attachment sites of KNorFES were the two lysine ϵ-amino groups (the P_6 and P_4' residues). Additionally, spectral properties of KNorFES homodoubly labeled with two different geometric isomers of DER were evaluated for insight into intramolecular H-dimer stereochemistry.

Model Structures. In Figure 1 representative energy-minimized structures for (a) TMR−NorFES−TMR, (b) TMR−NorFES−hex−TMR, (c) TMR−hex−NorFES−TMR, and (d) TMR−hex−NorFES−hex−TMR are presented. (For all studies reported here, only the 5-TMR isomer was used.) Using Discover 3.0, energy minimization of these NorFES analogues was carried out by setting the intramolecular distance between the nitrogens of the two amide bonds that connect the dyes to the substrates constant at 24 Å. This value was used since the absorption spectra of all three extended doubly labeled peptides had blue-shifted absorption peaks (Figures 3b−d) and fluorescence quenching similar to those that we reported earlier for TMR−NorFES−TMR.[1,7−9] In the first of this series of papers based on the spectroscopic data, we proposed a structure in which the two rhodamines were placed in a side-by-side configuration with a calculated point−dipole distance of ca. 6.1 Å. In addition to the energy-minimized doubly labeled substrates in Figure 1a, a second set of conformational energy minimization calculations was carried out for the same four substrates but without fluorophores or the 24 Å distance restriction; the results for this set of calculations are listed in Table 1. Figure 2 shows the number of derived conformational energy-minimized structures for the parent NorFES peptide without either the nitrogen-to-nitrogen intramolecular distance constraint stated above or the presence of fluorophore moieties; the derived distances and their corresponding conformational energy levels are plotted for each structure.

Spectroscopic Data. The absorption spectrum of TMR in solution at concentrations less than 1×10^{-5} M as well as for singly labeled peptides has a major peak at 552 (an extinction coefficient of ca. 9×10^5/M·cm) and a shoulder at 518 nm (Figure 3e); this spectrum together with a fluorescence quantum yield of ca. 0.9 (λ_{ex}=552; λ_{em} = 580) can be considered as the TMR monomer's optical signature. Formation of an H-dimer between two TMRs is known to distort these spectral characteristics such that the absorbance peak shifts to ca. 518 nm with a shoulder at ca. 552 nm concomitant with quenching of the

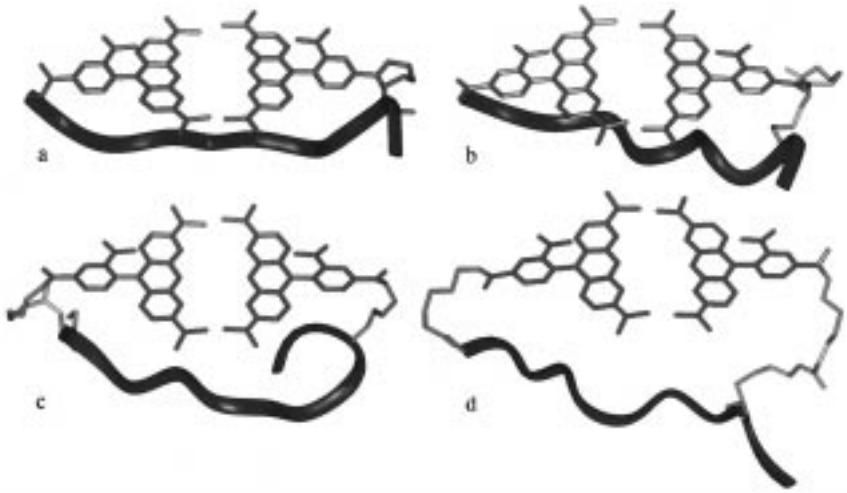

Figure 1. Model structures for (a) TMR−NorFES−TMR, (b) TMR−NorFES−hex−TMR, (c) TMR−hex−NorFES−TMR, and (d) TMR−hex−NorFES−hex−TMR. Structures were simulated using Discover 3.0 starting with a fixed intramolecular distance of 24 Å between the nitrogens of the two amide bonds connecting the dyes to the substrates. The red color represents the TMRs, blue the peptide backbone, and green the 6-aminohexanoic acid linker arms.

TABLE 1: Calculated Intramolecular Distances between Nitrogens of Unlabeled Substrates[a]

peptide	N−N distance (Å)	±SD (Å)
NorFES	13.56	4.63
NorFES−hex	15.60	4.59
hex−NorFES	18.07	5.43
hex−NorFES−hex	17.75	5.38

[a] Energy minimizations were carried out using Discover 3 for NorFES, NorFES−hex, hex−NorFES, and hex−NorFES−hex in the absence of any N−N distance constraints or fluorophores.

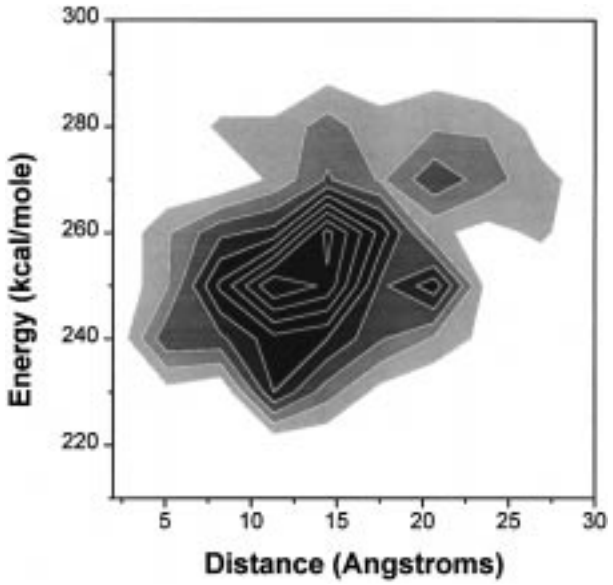

Figure 2. Plot of the number of NorFES structures with each N−N distance and the corresponding energy. The magnitude of the number increases from white to black.

fluorescence; these data are consistent with the monomer being fluorescent and the dimer nonfluorescent. To determine the effect of the addition of length and flexibility in the backbone on intramolecular dye−dye association including the free energy of activation ($\Delta G^{\ddagger}$) for disruption of the intramolecular dimer structures, absorption and fluorescence emission spectra of the four doubly labeled NorFES peptides were taken at 5° intervals ranging from 5 to 50 °C, and these spectral parameters were followed.

In Figure 3 the observed absorption spectra with maxima at ca. 518 nm and shoulders at ca. 552 nm are consistent with the presence of H-dimers to a significant extent in all four homodoubly labeled peptides. As the temperature was raised from 5 to 50 °C, decreases in the A_{518}:A_{552} ratios together with fluorescence increases in emission spectra (Figure 4) were observed. These changes were consistent with disruption of the intramolecular dimers as the temperature was increased.

The data plotted in Figure 4 represent the fluorescence of each doubly labeled peptide as a fraction of the total fluorescence of a solution containing an equal concentration of TMR conjugated to singly labeled peptides. Thus, the control against which these data were normalized was a solution containing completely cleaved TMR−NorFES−TMR. After the temperature study was completed, elastase was added to the cuvette containing each of the four doubly labeled intact peptide solutions at 37 °C. Following complete substrate cleavage in each solution the total fluorescence intensity in each cuvette was determined and used for a quantitative comparison among the four NorFES analogues.

As shown in Figure 4, the smallest change in fluorescence as a function of increasing the temperature from 5 to 50 °C was seen for TMR−NorFES−TMR. As expected, when one or two linkers was added, the temperature effect was greater. Interestingly, addition of the linker at the ϵ-amino group of the lysine (P_4' residue) side chain resulted in less quenching than in the parent, i.e., TMR−NorFES−TMR. However, addition of the same linker at the α-amino group of aspartic acid (the P_5 residue), e.g., in TMR−hex−NorFES−TMR, resulted in a greater degree of quenching than in the parent. In fact, addition of the linker at the amino terminus seemed to be critical for enhancing intramolecular dimer formation as both absorption and fluorescence data of TMR−hex−NorFES−TMR and TMR−hex−NorFES−hex−TMR were almost indistinguishable.

Thermodynamic Analysis. Using the fluorescence data presented in Figure 4, the activation free energies ($\Delta G^{\ddagger}$) listed in Table 2 were calculated from van't Hoff plot analyses for data between 25 and 50 °C (Figure 5). In addition to these $\Delta G^{\ddagger}$'s, which are free energies of activation for disruption of intramolecular dimers, the percent fluorescence quenching for each doubly labeled peptide at 37 °C is also given. The equilibrium constant K for the disruption of intramolecular

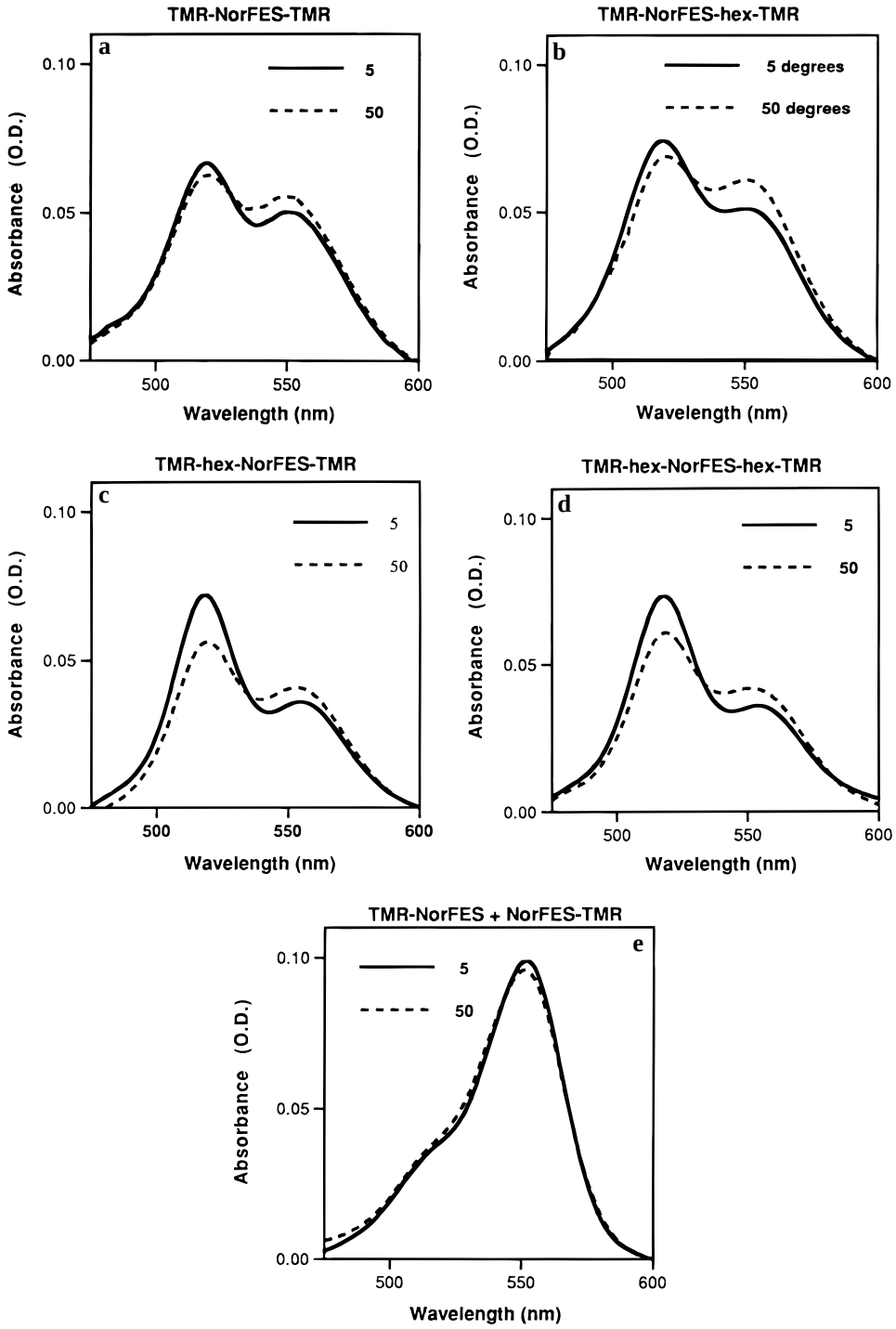

Figure 3. Absorption spectra at 5 °C (solid lines) and 50 °C (dashed lines) of (a) TMR−NorFES−TMR, (b) TMR−NorFES−hex−TMR, (c) TMR−hex−NorFES−TMR, (d) TMR−hex−NorFES−hex−TMR, and (e) TMR.

H-dimers at each temperature was determined by taking the ratio of mole fractions for nonfluorescent and fluorescent species, with the assumption that the dimer is nonfluorescent. For example, at 25 °C the fluorescence of TMR−NorFES−TMR is 9.75% that of the control solution (the fully cleaved peptide); hence, the ratio of the fluorescent to nonfluorescent species' mole fraction is 0.0975:0.9025, and the corresponding equilibrium constant for the formation of two fluorescent monomers from each nonfluorescent intramolecular dimer is 0.108.

KNorFES. Since the addition of a 6-aminohexanoic acid to the amino terminus resulted in the highest dimer:monomer ratio and percent quenching, e.g., in TMR−hex−NorFES−TMR and TME−hex−NorFES−hex−TMR, we synthesized TMR−KNorFES−TMR where NorFES is extended at its amino terminus by a lysine residue; the two TMRs were then conjugated via the ϵ-amino groups of the two lysines, i.e., the P_6 and P_4' residues. As the chemical structure of 6-aminohexanoic acid differs from that of an amino terminal lysine residue only by the presence of an amino group on the α-carbon of the latter, displacement of the new fluorophore attachment site is the same for both of these NorFES derivatives. The presence of this α-amino group at most could only add a small degree of steric hindrance in KNorFES relative to the hydrogen in hexNorFES. Consistent with this expectation, both substrates exhibit similar absorption spectra (Figures 6a and 3c) with a slightly decreased A_{518}:A_{552} ratio observed in TMR−KNorFES−TMR.

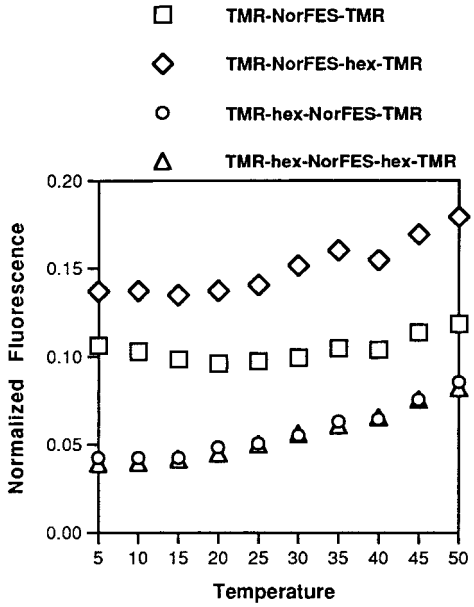

Figure 4. Normalized fluorescence intensity relative to completed cleaved peptides from 5° to 50 °C for TMR−NorFES−TMR (□), TMR−NorFES−hex−TMR (◇), TMR−hex−NorFES−TMR (○), and TMR−hex−NorFES−hex−TMR (△).

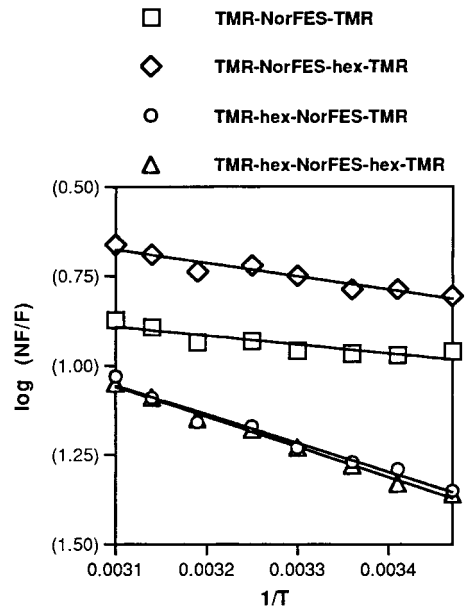

Figure 5. van't Hoff plots of TMR−NorFES−TMR (□), TMR−NorFES−hex−TMR (◇), TMR−hex−NorFES−TMR (○), and TMR−hex−NorFES−hex−TMR (△) for temperatures between 15° and 50 °C.

TABLE 2: Free Energies of Activation ($\Delta G^{\ddagger}$) and Percent Quenching (%Q) at 308 K of Intact Substrates Relative to Completely Cleaved Substrates[a]

peptide	$\Delta G^{\ddagger}_{308}$ (cal/mol)	$\%Q_{308}$
TMR−NorFES−TMR	1316	90
TMR−NorFES−hex−TMR	1035	84
TMR−hex−NorFES−TMR	1659	94
TMR−hex−NorFES−hex−TMR	1666	94

[a] $\Delta G^{\ddagger}$ values were calculated from data in van't Hoff plots of Figure 5.

Comparison of TMR Dimers with DER Dimers. DER has the essential structural properties that result in H-dimer formation as previously described, i.e., delocalized charge, symmetry, and high transition dipole.[7] In addition, replacement of the two

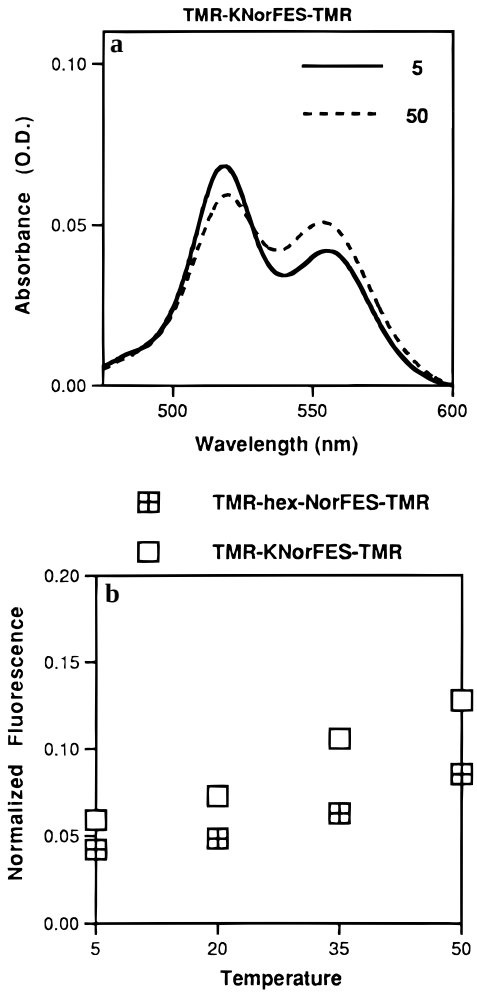

Figure 6. (a) Absorption spectra at 5 °C (solid line) and 50 °C (dashed line) of TMR−KNorFES−TMR. (b) Normalized fluorescence intensity relative to completed cleaved peptides from 5° to 50 °C for TMR−KNorFES−TMR (open boxes) and TMR−hex−NorFES−TMR (crossed boxes).

methyl groups on each of TMR's xanthene nitrogens with one ethyl group and one hydrogen atom in DER allowed us to probe the steric effects in the side-by-side configuration of our proposed intramolecular H-dimer model.[1] One would predict that substitution on the xanthene nitrogens could impact significantly on dimerization if the dyes were side-by-side; in contrast, if the dyes were stacked, the steric effects would be negligible. We therefore homodoubly labeled KNorFES with the 5-isomer of each of these rhodamines and compared absorption spectra and fluorescence intensity as a function of temperature. Additionally, KNorFES was also homodoubly labeled with 6-DER. In Figure 7 quenching of the two DER derivatives is shown to be 3−6 times greater than that of the same peptide (KNorFES) homodoubly labeled with 5-TMR from 5 to 50 °C. Consistent with these data are the higher dimer: monomer ratios, i.e., the A_{500}: A_{527} ratios, shown in Figure 8a,b. The thermodynamic parameters obtained from van't Hoff analyses of the fluorescence data listed in Table 3 indicate that both DER homodimers are more stable than that formed by TMR as judged by the greater barrier height for disrupting the intramolecular dimers. Interestingly, the calculated barrier height difference of ca. 300 cal/mol between the 5- and 6-DER homodoubly labeled peptides is similar to that between TMR−NorFES−TMR and TMR−hex−NorFES−TMR or TMR−hex−NorFES−hex−TMR. These values together with the proposed structures for 5-TMR-KNorFES−5-TMR, 5-DER−

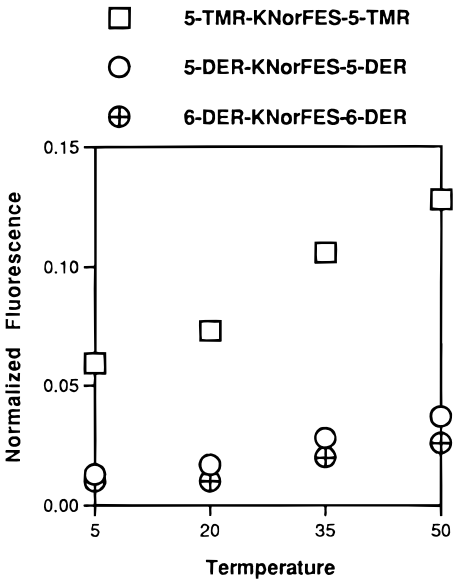

Figure 7. Normalized fluorescence intensity relative to completed cleaved peptides from 5° to 50 °C for TMR−KNorFES−TMR (□), 5-DER−KNorFES−5-DER (○), and 6-DER−KNorFES−6-DER (⊕).

TABLE 3: Free Energies of Activation ($\Delta G^{\ddagger}$) and Percent Quenching (%Q) at 308 K of Intact Substrates Relative to Completely Cleaved Substrates

peptide	$\Delta G^{\ddagger}_{308}$(cal/mol)	$\%Q_{308}$
5-TMR−KNorFES−5-TMR	1343	90
5-DER−KNorFES−5-DER	2214	97
6-DER−KNorFES−6-DER	2459	98

KNorFES−5-DER, and 6-DER−KNorFES−6-DER as shown in Figure 9 suggest a closer approach of the two DER dyes as compared with two TMRs; this putative steric difference between DER−DER and TMR−TMR dimers is believed to result from the variation in xanthene ring nitrogen substituents.

Discussion

We have previously shown that with the principles of exciton theory as a guide very effective profluorescent protease substrates can be made by doubly labeling polypeptides that contain sequence information from both sides of protease recognition and cleavage sites.[1,2] Moreover, the dequenching that results from separation of the two fluorophores to effectively infinite distance by a protease-induced cleavage of a substrate's peptide backbone can be used to quantitate proteolytic activity. The overall objective of the work described here was to augment understanding of the interactions between dyes that lead to intramolecular excitonic H-type dimers. The approach taken was to evaluate the orientation of dyes in dimeric structures using different peptide and dye-linker arms as well as two different geometric fluorophore isomers. Thus, one aim of this work was to determine the free energy of activation ($\Delta G^{\ddagger}$) associated with the disruption of intramolecular excitonic H-type dimers using the spectroscopic data of these derivatives. This calculated value serves as an upper estimate for the magnitude of free energy of dimer stabilization. A second aim was to evaluate how altering the length, flexibility, and stereochemistry of the backbone dye-linker moiety impacts on the stability of this type of dye−dye dimer as judged by the calculated differences in barrier height for dimer disruption. A third aim was to seek further insight into the planar side-by-side intramolecular dye−dye dimer structure that we proposed earlier.[1]

NorFES is an undecapeptide (DAIPN$_l$SIPKGY, N$_l$ = norleucine) containing a cleavage recognition site for the serine protease elastase. In antecedent studies,[7−9] we showed that although doubly labeling this sequence with a variety of fluorophores results in several different elastase substrates, each with its own spectroscopic and biochemical properties, the most efficiently quenched substrates were those in which a symmetric fluorophore with a delocalized charge and a strong transition dipole, e.g., a rhodamine, was bound to both sides of the peptide's cleavage site. The spectral characteristics of these substrates are consistent with the exciton model for H-dimer formation;[3−6] in the latter a correlation exists between the degree of quenching and the percent blue-shifted peak in absorption spectra. Since in our previous work TMR showed the optimal characteristics of six dyes compared,[7] this was the first dye selected for the current study.

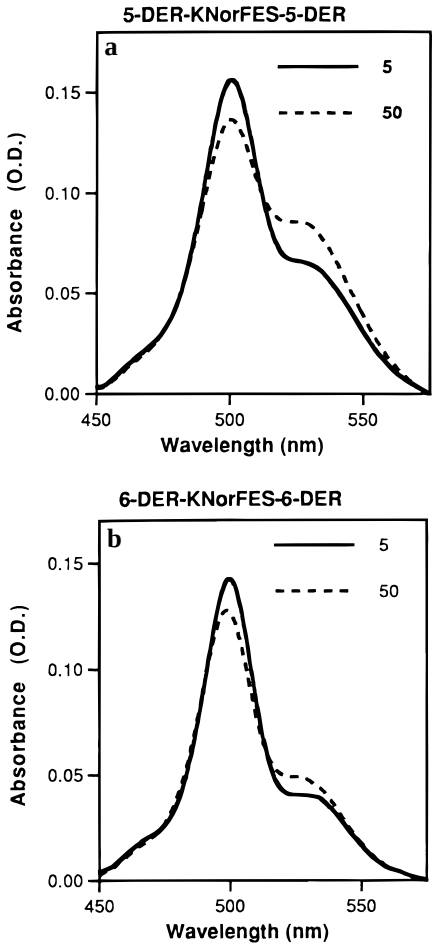

Figure 8. Absorption spectra at 5 °C (solid lines) and 50 °C (dashed lines) of (a) 5-DER−KNorFES−5-DER and (b) 6-DER−KNorFES−6-DER.

Addition of Flexible Linker Arms to the Peptide Backbone. To make an initial assessment of the influence of backbone length and flexibility on intramolecular H-dimerization, spectral properties among NorFES derivatives where the peptide backbone was extended by addition of one or two 6-aminohexanoic acids (hex) were compared (Figures 3 and 4). In our previously proposed model structure for TMR−NorFES−TMR the two rhodamines were in a side-by-side configuration with a distance of 24 Å between the two amide nitrogens joining the dyes to the backbone.[1] Existence of the ca. 518-nm peak in the absorption spectra of the four doubly labeled peptides in the current study (Figures 3a−d) was consistent with similar dye−dye orientations for each substrate. Hence, structures presented in Figure 1 were derived by assuming an intramolecular distance of 24 Å between the two terminal amide

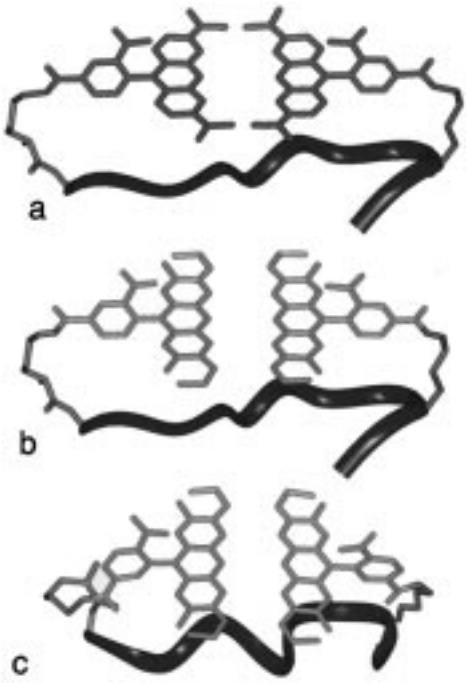

Figure 9. Model structures for (a) TMR−KNorFES−TMR, (b) 5-DER−KNorFES−5-DER, and (c) 6-DER−KNorFES−6-DER. The red color represents the TMRs, pink the DERs, and blue the backbone moieties.

nitrogens where each dye was coupled either directly to the backbone or via a linker arm.

The presence of an H-dimer in each peptide was examined by comparing this 24 Å distance with the ranges of N−N distances for the four peptides where energy minimization was carried out in the absence of both N−N distance constraints and fluorophores; these data are listed in Table 1. Interestingly, the conformational energy-minimized structures for the parent peptide substrate alone, NorFES, showed an average N−N distance considerably shorter (13.6 ± 4.6 Å) than the 24 Å required for insertion of the planar side-by-side H-dimer (Figure 1a). In Figure 2 the number of conformational energy-minimized structures of NorFES with the indicated N−N distance and corresponding conformational energy is plotted. It is quite apparent from this figure that in order to accommodate an intramolecular H-dimer in NorFES, the peptide backbone conformation needs to stretch. In doing so, one would predict the conformational energies for the stretched structures with the N−N distance approximating 24 Å to be higher than the averaged conformational energy-minimized structures of the peptides without dyes (Figure 2). Accordingly, addition of a flexible linker arm, e.g., a hex moiety, would be expected to reduce the strain associated with the introduction of an H-dimer; one would predict that reduction of such unfavorable conformational energy contributions to the free energy of dimer formation would result in an increase in the equilibrium constant for H-dimer formation. Thus, one would expect increases in both the A_{518}:A_{552} ratio and fluorescence quenching.

Spectroscopy of Intramolecular Dimers and Monomers. In addition to the molecular dynamics simulations and the conformational energy-minimization calculations, which suggest a need for peptides to stretch in order to accommodate an H-dimer, the spectroscopic data support roles for peptide conformational constraint and backbone entropy in determining the amount of H-dimer formation. Specifically, the absorption spectra of the four NorFES peptides in Figure 3 all show peaks at ca. 518 nm, indicative of the presence of H-dimers to a significant degree, and shoulders at ca. 552 nm (552 nm is not only the peak of the TMR monomers spectrum but also a shoulder in the TMR−TMR dimer spectrum). The highest A_{518}:A_{552} ratio is observed for hex−NorFES−hex and hex−NorFES, followed by NorFES, and finally NorFES−hex. The data in Figure 4 show the same order of fluorescence quenching at temperatures ranging from 5 to 50 °C. Earlier we demonstrated that by superimposing absorption and excitation spectra of TMR−NorFES−TMR fluorescence is lost upon dimerization.[1] Moreover, the shape of the fluorescence emission spectrum of a solution containing TMR−NorFES−TMR is identical to that of a solution of TMR at concentrations less than 1×10^{-5} M. Hence, the spectral data propound that addition of a flexible arm to the relatively rigid amino terminus significantly enhances intramolecular dimerization whereas the same addition to the ϵ-amino group of lysine at P_4' does not. The latter effect is most likely a result of the relative freedom of motion of the amino group of lysine, which is intrinsic to this four-methylene-containing amino acid side chain.

Relative Stability of Intramolecular H-Dimers in NorFES Peptide Analogues. To compare the stability of the four homodoubly labeled peptides, a thermodynamic analysis was carried out using the spectroscopic data obtained for each substrate between 15 and 50 °C. The model used was that of an equilibrium between nonfluorescent (NF) and fluorescent (F) species, i.e.,

$$\mathrm{NF} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{F} \tag{1}$$

$$K = \frac{\mathrm{F}}{\mathrm{NF}} \tag{2}$$

where NF is represented by the percent quenching and F by the percent fluorescence. The magnitudes of the $\Delta G^{\ddagger}$'s and percent quenching listed in Table 2 for each homodoubly labeled peptide support the predominance of a nonfluorescent dimer in each substrate; moreover, the decreasing magnitudes of these free energies of activation suggest the relative order of dimer stability to be: TMR−hex−NorFES−hex−TMR ∼ TMR−hex−NorFES−TMR > TMR−NorFES−TMR > TMR−NorFES−hex−TMR. Since the largest barrier to disruption is ca. 1.7 kcal/mol, one would expect the magnitude of the free energy for H-dimer formation to be at most this value, on the basis of a totally nonfluorescent dimer. It is quite interesting to note the significant effect of flexible linker arm addition at the α-amino group compared with addition at the ϵ-amino group of lysine at P_4'. Comparing the activation free energies for TMR−NorFES−TMR, TMR−hex−NorFES−TMR, and TMR−hex−NorFES−hex−TMR, one notes the addition of a second linker arm at lysine p_4' has virtally no effect. This trend is consistent with the calculated N−N distance for NorFES, hex−NorFES, and NorFES−hex (13.6 ± 4.6, 18.1 ± 5.4, and 15.6 ± 4.6, respectively).

Replacement of a Linker Arm with an Amino Acid. Since addition of a flexible linker arm to NorFES's amino terminus resulted in the most favorable conditions for intramolecular dye−dye dimerization, we replaced this six-carbon arm with a lysine, an amino acid that can be correctly called 2,6-diaminohexanoic acid. The absorption and fluorescence properties of this doubly labeled peptide, i.e., TMR−KNorFES−TMR, were then compared with those of TMR−hex−NorFES−TMR. Since the two peptides differ only by the addition of an amino group on the a carbon of the lysine at the N-terminus of KNorFES, the lengths of the two backbones are the same and

the flexibilities would be expected to be quite similar. Indeed, both substrates exhibit similar absorption spectra (Figures 6a and 3c) and fluorescence intensities (Figure 6b) with a slightly decreased A_{518}:A_{552} ratio in TMR−KNorFES−TMR.

TMR vs DER. In our previously proposed model structure for TMR−NorFES−TMR, the two rhodamines are side-by-side with the point−dipole separation estimated to be ca. 6.1 Å. This distance was determined by using the equation

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

where

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

and θ_{12}, θ_{1R}, and θ_{2R} 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. In the case of H-dimers, $\theta_{12} = 0$, $\theta_{1R} = \theta_{2R} = 90°$, which yields $\kappa = 1$, whereas for J-dimers $\theta_{12} = \theta_{1R} = \theta_{2R} = 0°$, which yields $\kappa = -2$. U was calculated from the blue-shifted absorbance peak of 518 nm relative to the monomer peak at 552 nm. As stated above, among TMR doubly labeled substrates the spectral differences are not in the size of the blue-shift, i.e., it is ca. 34 nm in each, but in the relative peak heights at 518 and 552 nm.

Physical Characterization of Intramolecular Ground-State Dimers. From the model structures in Figure 1, the potential for steric hindrance in H-dimerization arising from the four methyl groups of each xanthene of TMR is apparent. To better assess this possibility, the spectral properties of KNorFES homodoubly labeled with 5-DER were compared with those of the same peptide homodoubly labeled with 5-TMR. An increased percentage of molecules exhibiting both an absorbance blue-shift (Figures 8a) as well as increased fluorescence quenching (Figure 7) in 5-DER−KNorFES−5-DER compared with 5-TMR−KNorFES−5-TMR (Figures 6a and 7) was observed. Moreover, the increase in magnitude of $\Delta G^{\ddagger}$ from 1.3 to 2.2 kcal when comparing these two bifluorophoric substrates supports a stronger intramolecular association between DER dyes than between TMRs. Using eq 3 and the blue-shift of the DER monomer to dimer (from 527 to 500 nm), the distance between the centers of the point−dipole of the DER−DER dimer was calculated to be ca. 0.5 Å less than the previously calculated value for the TMR−TMR dimer. This calculated shorter distance is consistent with a decrease in steric hindrance between the dyes in the DER−DER dimer. The source of this reduction in steric hindrance is most likely due to the replacement of the two methyls per nitrogen in TMR with only a single ethyl and a hydrogen in DER. Overall, these data corroborate the side-by-side dimer configuration we proposed earlier over a stacked structure for intramolecular H-type dimers.

Interestingly, the higher percent dimer of the 6-DER isomer vs the 5-DER isomer, as determined by the higher percent quenching (Figure 7) and increased A_{500}:A_{527} ratio (Figure 8) together with the resultant slight increase in $\Delta G^{\ddagger}$ magnitude from 2.2 to 2.4 kcal (Table 3), are most likely due to the greater facility permitted by the carboxyl in the 6- vs the 5- position on the lower ring of the rhodamine. As illustrated in Figure 9, the 60° angle dye−peptide attachment orientation difference between the 6- and 5-isomers of DER would be expected to result in reduction of the required N−N distance by about 4 Å. Hence, insertion of an H-dimer with the 6-isomer geometry would be predicted to require less perturbation on the peptide moiety conformation. If the intramolecular dye−dye dimer structure were not the previously proposed side-by-side configuration but rather a dye−dye stacking configuration, then formation of the latter structure would not be affected by the stereochemistry of the peptide conjugation. Additionally, one would not predict a difference in the $\Delta G^{\ddagger}$ magnitudes between TMR−NorFES−hex−TMR and TMR−hex−NorFES−TMR since the required N−N distance of ca. 13 Å would already be fulfilled by the preferred conformational energy-minimized structures of the parent NorFES peptide, i.e., (13.6 ± 4.6) Å.

Conclusion. Overall the data presented in this paper show that extension of the backbone of TMR−NorFES−TMR, a doubly labeled profluorescent protease substrate, can affect the percentage of intramolecular H-dimer formation and, therefore, percent quenching of an intact substrate. The blue shifts in the absorption spectra of ca. 34 nm for TMR and 27 nm for DER derivatives as well as the correlations between A_{518}:A_{552} and A_{500}:A_{527} ratios, respectively, with percent quenching are consistent with essentially the same dimer structure in each doubly labeled NorFES substrate, regardless of the extension. Since similar spectral shifts are frequently observed in solutions of proteins labeled with xanthenes,[11−13] it is quite possible that the appearance of excitonic bands in spectral data, e.g., a 518-nm absorbance maximum in a solution containing a protein labeled with TMR, may indicate that a distortion of the native protein structure has been induced by the addition of this label unless the dye conjugation sites are about 24 Å apart.

Additionally, an inference regarding the nature of the dye−dye interaction in H-dimers can be drawn on the basis of the data presented in this paper. The decrease in the dimer: monomer ratio as the temperature is raised, particularly in NorFES derivatives with backbones extended by one or two flexible 6-aminohexanoic acids, points toward an intramolecular attraction between two dyes with an energy of activation for disruption of this interaction of at least 1.7 kcal/mol for TMR and 2.4 kcal/mol for DER. Finally, the observed effects of addition of a flexible linker arm or a lysine residue or substitution of one geometric DER isomer for another on the fluorescence quenching, absorbance ratios, and $\Delta G^{\ddagger}$'s are consistent with the side-by-side configuration of the previously proposed intramolecular H-dimer structure.

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