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Distance Distributions of Short Polypeptides Recovered by Fluorescence Resonance Energy Transfer in the 10 Å Domain

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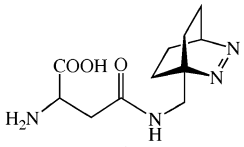
Fluorescence resonance energy transfer (FRET) in combination with Förster theory has become an invaluable tool for the assessment of distances in numerous biomolecular assemblies.[1,2] The "spectroscopic ruler" work in polyprolines presents a seminal study in this respect,[3] which confirmed the strong distance dependence (R^{-6}) of FRET. Different donor/acceptor pairs can be selected, and each possesses a characteristic Förster radius (R_0), which determines the distance domain accessible to FRET measurements. Numerous FRET pairs with R_0 values between 20 and 100 Å are available, such that distances in this range can be routinely analyzed.[1−3] Short flexible polypeptides with up to 20 amino acids in their backbone and an average end-to-end distance of 5−20 Å, however, would require donor/acceptor pairs with an R_0 value around 10 Å. Experimental data on average distances and distribution functions of such short peptides are quintessential for the improved parametrization of force fields for molecular dynamics (MD) simulations, which can nowadays be performed for polypeptides composed of up to 10−20 amino acids.[4,5] However, only case studies of such "short-distance" pairs have been reported, for instance, with 4-oxopentanoic acid (R_0 = 6.3 Å)[6] and 2-aza-2-deoxy-D-arabino-hexitol (R_0 = 9.8 Å)[7] as acceptor, which were limited to synthetic polymers and a singular example for a protein.

Herein, we introduce the use of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) as an acceptor which in combination with the intrinsic fluorescence of tryptophan (Trp) constitutes a convenient donor/acceptor pair with an R_0 value of 10 Å. This allows one to characterize average end-to-end distances and distribution functions in short flexible peptides by means of a combination of steady-state and time-resolved fluorescence and to compare them with those from MD simulations.

DBO



Dbo

The present study shows that DBO is not only itself an attractive fluorophore for protease assays[8] and for probing the dynamics of biomolecular processes[9,10] but also an interesting acceptor in FRET studies. Figure 1 shows the spectral properties relevant for the calculation of R_0. As can be seen, the fluorescence spectrum of Trp overlaps nicely with the absorption of DBO (λ_{max} = ca. 365 nm). Two special assets of the acceptor absorption spectrum stand out: (i) the optical window of the acceptor in the region of donor absorption (λ_{exc} = 280 nm), which allows a perfectly selective donor excitation, and (ii) the low extinction coefficient of DBO (ca. 50 M^{-1} cm^{-1} in water), which is related to the overlap-forbidden nature of the n,π^* transition. The latter leads to very small overlap integrals and, thus, a very short Förster radius (R_0 = 10 Å), ideally suited to resolve distance relationships in the 5−20 Å domain.

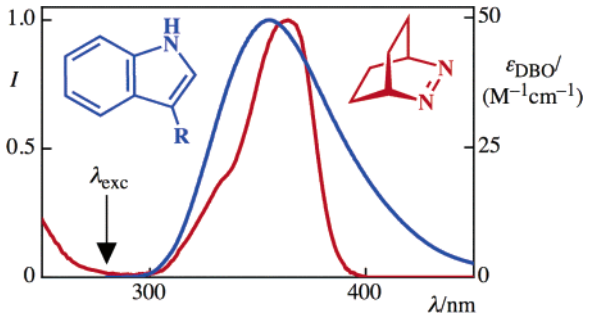


Figure 1. Spectral overlap for the Trp/Dbo FRET pair.

The point-dipole approximation made in Förster theory requires that the range of the investigated distances and therefore the R_0 value does not fall below the size of the donor and acceptor chromophores.[11,12] On the basis of the small size of the Trp donor and especially of the DBO acceptor, for which the electronic transition is localized on the two atoms of the azo group (N═N), the point-dipole approximation should hold at distances even as small as 5 Å. This contrasts classical polyaromatic donor/acceptor systems, including strongly absorbing dyes accessible to single molecule studies, where distances below 20 Å remain difficult to determine.[11,13] Further work has demonstrated that interferences due to exchange and other short-range interactions, including higher terms in the multipole expansion of the Coulombic interaction, present no principal obstacle to the classical dipole−dipole analysis of FRET data for short-lived donors at distances greater than 5 Å.[14] Such additional mechanisms would, in fact, accelerate energy transfer and lead to apparent distances which would be far too small when compared with theoretical expectations. However, our results show that exactly this is not the case.

The novel acceptor is readily introduced into polypeptides by using the known Fmoc-protected asparagine derivative Dbo in solid-phase synthesis.[9] For the present exploratory FRET study, we have selected flexible and structureless[9] Trp-(GlySer)$_n$-Dbo-NH_2 peptides with n = 0−10. The experimental FRET data (Table 1) were measured in three different solvents (water, ethanol, and propylene glycol) by both steady-state as well as picosecond time-resolved fluorescence spectroscopy (averaged donor fluorescence lifetimes were used). We restricted the detailed analysis to propylene glycol, where diffusion of the chain ends and the associated diffusion-enhanced FRET is negligible due to the 45 times higher viscosity than water. In fact, end-to-end diffusion for the investigated peptides has been recognized to be relatively slow even in water (10−100 ns)[9] in comparison to the Trp excited-state lifetime in the peptides (ca. 0.4−2.6 ns), but was nevertheless found to increase the FRET efficiency slightly in nonviscous solvents (cf. Supporting Information). Note that the energy transfer efficiency of the Trp-Dbo-labeled peptides, determined from the decrease in donor fluorescence, fell in the ideal range of 10−90%, which reveals immediately the

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Table 1. FRET Measurements and Recovered Distributions

n	E_{ss}[a]	*F*[b]	l_p/Å[c]	l_c/Å[c]	R_{avg}/Å[d]	R_{MD}[e]
0	0.72	0.12	46.5	9.6	8.7	8.6
1	0.56	0.16	13.8	11.5	9.0	8.0
2	0.45	0.13	12.1	12.6	9.7	8.2
4	0.32	0.11	11.4	14.9	11.0	9.5
6	0.24	0.06	10.0	16.2	11.7	10.2
10	0.12	0.04	10.4	18.8	13.7	

[a] Steady-state energy transfer efficiency. [b] Fraction of static quenching, calculated as $F = E_{ss} - E_{tr}$, where E_{tr} is the energy transfer efficiency obtained from time-resolved fluorescence (average donor lifetimes). [c] Persistence length and contour length from fitting according to wormlike chain model; 5% error. [d] Average distance including contact conformations. [e] Average distance between the centers of the indole ring and the azo group obtained from MD simulations (GROMOS96 force field, ref 17).

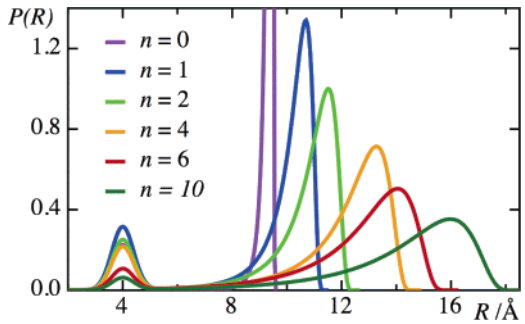

Figure 2. Distribution functions recovered from a combined analysis of steady-state and time-resolved FRET efficiencies; function for $n = 0$ is cut off for clarity; the short-distance peak represents the static quenching component and was placed at 4 Å (1.0 Å half-width).

advantage of using a short-distance FRET pair for investigating such short peptides. The study could therefore be extended even to the shortest dipeptide, where FRET is most efficient but not quantitative.

The steady-state FRET efficiencies (E_{ss} in Table 1) were in all cases significantly larger than those extracted from the time-resolved measurements, which is in line with a static quenching component, that is, a small part of the sampled conformations is already in donor−acceptor contact when excitation takes place. Excitation of these preformed encounter complexes is expected to result in immediate energy transfer because in addition to the dipole−dipole the Dexter exchange mechanism operates at contact distance (<5 Å). The fraction of static quenching (*F* in Table 1, taken as difference between the steady-state and time-resolved FRET efficiencies) was in the range of 10%.

Recent MD simulations[4,5] have indeed suggested multimodal distributions, similar to those shown in Figure 2, with a characteristic narrow peak at a short distance of 4.0 Å (half-width ca. 1 Å) corresponding to peptide conformations in which the chain ends are in van der Waals contact. Accordingly, we assign the static quenching component to the short-distance "hump" of the distribution functions to be recovered. Following a method originally devised by Cantor and Pechukas,[15] we assessed the second (noncontact) part of the distribution function by fitting a distribution-dependent FRET kinetics to the time-resolved fluorescence decays; the latter reflects the decay of the conformations with separations larger than 5 Å. The distribution functions were modeled with a Gaussian chain (Supporting Information) and a wormlike chain (Table 1).[16] The latter afforded distribution functions characterized by the contour length (l_c) as a measure of the chain length and the persistence length (l_p) as a measure of the chain stiffness and width of the distribution. In addition, average distances (R_{avg}) were derived from the net distribution functions; these include the contribution of the short-distance component, see Figure 2, to allow a direct comparison with MD simulation data.

The contour lengths of the recovered distribution functions increased gradually with peptide length from 9.6 to 18.8 Å, while the persistence length showed a decrease with increasing length, leveling off toward 10 Å. The recovered persistence length is characteristic for semi-flexible biopolymers (ca. 10−14 Å),[16] except for the shortest one (46.5 Å). The latter does, in fact, not contain the flexible glycine−serine linkage and is known to display an unexpectedly slow loop formation rate in independent kinetic experiments.[9] Interestingly, the recovered R_{avg} values lie above the average distances projected from MD simulations (R_{MD}, Table 1). The deviation is rather systematic (ca. 1−1.5 Å, except for $n = 0$), which may be due to the employed force field (GROMOS96).[5,17]

In summary, the Trp/Dbo FRET pair presents a unique and experimentally convenient handle to evaluate distribution functions for short flexible peptides. These constitute benchmarks for the refinement of force fields and the modeling of larger proteins. Apart from affording absolute distances, the FRET pair has similar potential for probing relative proximity relationships and structural transitions in the 10 Å domain.

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Supporting Information Available: Details on FRET analysis and MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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