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Distribution of distances in thiopeptides by fluorescence energy transfer and frequency-domain fluorometry *

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Frequency-domain fluorescence spectroscopy was employed to examine the decays of tryptophan in Boc-Trp-Met-Asp-Phe-NH_2 (donor) and Boc-Trp-Met-Asp-Phe-C(=S)-NH_2 (donor-acceptor pair). The efficiency of energy transfer in the thiopeptide amounted to 60%. The measured dispersion of fluorescence decay times was used to recover the donor–acceptor distance distribution. The parameters of the Gaussian distance distribution obtained for this peptide ($\bar{r}$, the mean distance (9 Å); hw, the halfwidth (25 Å)) indicate the lack of a distinct favorable conformation.

1. Introduction

In recent years, numerous investigations have been carried out on intramolecular excitation energy transfer in natural peptides [1–3], as well as in peptides modified with acceptor groups dinitrophenyl, dansyl, etc.) [4–6]. These studies revealed the efficiency of energy transfer, E, and the corresponding average donor separation to be determined. However, for many peptides one does not expect a single conformation, and hence the average distance represents a weighted average over the range of available conformations. A few pioneering studies, predominantly in model systems in which donor and acceptor moieties were linked with a polymethylene or polypeptide chain, have been reported by Steinberg and co-workers [7,8], Fung and Stryer [9] and Lakowicz and co-workers [10]. The main difficulty in recovering a distribution of distances is that one requires high-quality data in either the time or frequency domain. An additional difficulty is found with short peptides, which can result in a high transfer efficiency and considerable error in the determination of the donor-acceptor separation, $\bar{r}$ [11].

During the past few years, a method has been developed for the synthesis of peptides containing the thiocarbamide group, with the resulting peptides exhibiting potent biological activity. Relatively few papers have appeared on the structure

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* Dedicated to Professor Joseph R. Lakowicz on the occasion of his 40th birthday

Abbreviations: t-Boc; *N-tert*-butoxycarbonyl; DCC, dicyclohexylocarbodiimide; HBOT, *N*-hydroxybenzotriazole; Lawesson's Reagent, (2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide; Phet, phenylalanine thioamide.

Boc-Trp-Met-Asp-Phe-NH_2

Donor

Boc-Trp-Met-Asp-Phe-C(=S)-NH_2

D-A Pair

Scheme 1. Donor and donor-acceptor peptide structures.

and function of thiopeptides, five of which are known to display biological activity [12–17]. These reports indicate that thioamide bonds in the peptide backbone or C-terminal part of biologically active peptides can drastically affect receptor affinity and selectivity, as well as biological potency. The considerable similarity between amides and thioamides [18] and a possible increase in susceptibility to enzyme degradation (refs. 19–22; and M. Kruszynski and G. Kupryszewski, manuscript in preparation) can be essential determinants in using thioamide-modified peptide hormones.

The aim of the present paper is to investigate the intramolecular excitation energy transfer from tryptophan to the thiocarbonyl bond. The donor and the D-A pair are depicted in scheme 1.

Since the linker is partially flexible, a range of D–A distances is expected. The dispersion of the D–A distances results in dispersion of the transfer rates and, hence, of the frequency response of the donor. The acceptor-induced decrease in decay time and heterogeneity in the donor decay were used to recover the probability distribution of the tryptophan–thiocarbonyl distance.

2. Theory

The intensity decays of most peptides and proteins containing a single tryptophan residue are usually multiexponential [23–26]. The heterogeneity of the donor decay and that of the donor-acceptor system are given by the following expressions [27].

$$I_D(t) = \sum_i \alpha_{D_i} \exp(-t/\tau_{D_i}) \tag{1}$$

$$I_{DA}(r,t) = \sum_i \alpha_{D_i} \exp\left[-t/\tau_{D_i} - \frac{t}{\tau_{D_i}}\left(\frac{R_0}{r}\right)^6\right] \tag{2}$$

where α_{D_i} are the pre-exponential factors and τ_{D_i} the associated decay times for the donor in the absence of an acceptor. Eq. 2 describes the intensity decay of a single donor-acceptor pair separated by a distance r. In writing eq. 2 we assumed that the transfer rate from each component in the decay is given by $\tau_{D_i}^{-1}(R_0/r)^6$. R_0 is the Förster distance [28] which can be calculated from spectral properties of the chromophores

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\phi_D^0}{128\pi^5 N n^4} \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda \tag{3}$$

where κ^2 is the orientational factor, n the refractive index, N Avogadro's number, ϕ_D^0 the quantum yield of the donor in the absence of the acceptor, $F_D(\lambda)$ the emission spectrum of the donor, the area being normalized to unity and $\epsilon_A(\lambda)$ the extinction coefficient (in $M^{-1}\,cm^{-1}$) of the acceptor at wavelength λ (in nm). The calculation of distances also requires some knowledge of κ^2 which can be obtained from measurements of the emission anisotropy [29]. In our analysis we have set $\kappa^2 = 2/3$. This is justified by the possibilities of rotational diffusion, the range of conformations and the mixed polarization of the chromophores [29,30]. The intensity decay of an ensemble of D-A pairs is given by

$$I_{DA}(t) = \int_0^\infty P(r) I_{DA}(r,t) \, dr \tag{4}$$

In this report we assume a Gaussian probability distribution

$$P(r) = \frac{r^m}{\sigma\sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{r-\bar{r}}{\sigma}\right)^2\right] \tag{5}$$

where $\bar{r}$ is the mean distance, σ the standard deviation ($\sigma = hw/2.354$) and hw the full-width at half-maximum. The choice of m depends upon whether the distance distribution is assumed to be Gaussian along a line ($m = 0$) in a plane ($m = 1$) or in space ($m = 2$). As has been found in previous investigations [10,27], the recovered distributions were visually similar where m was 0, 1 or 2.

With frequency-domain fluorometry, one measures the frequency (ω) dependent values of the phase (Φ_ω) and modulation (m_ω). The values of r and hw are calculated by least-squares fitting of

the measured values to those calculated ($\Phi_{\omega c}$, $m_{\omega c}$). For the distance distributions, numerical integration was employed to calculate the sine and cosine transforms of eq. 4,

$$N_\omega = \int_0^\infty \sum_i \frac{P(r)\omega\tau_{DA_i}^2}{1+\omega^2\tau_{DA_i}^2} dr \quad (6)$$

$$D_\omega = \int_0^\infty \sum_i \frac{P(r)\tau_{DA_i}}{1+\omega^2\tau_{DA_i}^2} dr \quad (7)$$

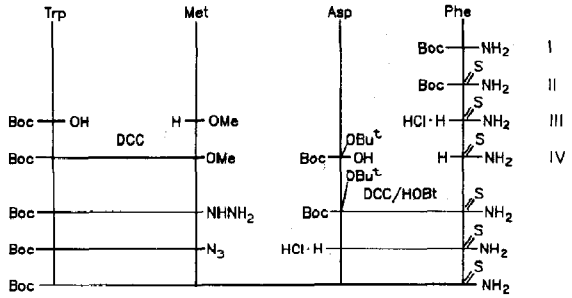
The values of the parameters were determined by minimizing the best-fit parameter

$$\chi_R^2 = \frac{1}{\nu}\sum_\omega \left[\frac{\Phi_\omega - \Phi_{\omega c}}{\delta\Phi}\right]^2 + \frac{1}{\nu}\sum_\omega \left[\frac{m_\omega - m_{\omega c}}{\delta m}\right]^2 \quad (8)$$

where ν is the number of degrees of freedom and $\delta\Phi$ and δm the experimental uncertainties in the measured phase and modulation values, respectively. The uncertainties in $\bar{r}$ and hw are estimated with consideration of correlation between the parameters [31,32]. It should be noted that the use of a multiexponential model to describe the donor decay (eq. 1) does not introduce additional variable parameters into the distance distribution analysis (eqs. 4–8), because the donor decay parameters (α_{D_i} and τ_{D_i}) are fixed parameters in the latter analysis.

3. Materials and methods

Synthesis of the N-protected C-terminal tetrapeptide sequence of gastrin with a C-terminal thioamide group (the acceptor) was performed according to scheme 2: *tert*-Butoxycarbonyl-L-phenylalanine thioamide (Boc-Phet-NH_2) and its derivatives (HCl · Phet-NH_2 and Phet-NH_2) were obtained according to a known procedure [16] using Lawesson's Reagent as the thionation agent [33]. The structural proofs of compounds I–IV were based on elemental analysis, and ^{1}H-NMR and ^{13}C-NMR spectroscopy. All other intermediates (Boc-Trp-Met-OMe and Boc-Trp-Met-$NHNH_2$) and the reference compound (Boc-Trp-Met-Asp-Phe-NH_2) were synthesized as described elsewhere [34,35]. L-Phenylalanine thioamide (IV) was coupled with Boc-Asp (OBu^t) using the



Scheme 2. Synthesis of Boc-Trp-Met-Asp-Phe-NH_2 (Phet is used to indicate the thiocarbonyl analog of a phenylalanine residue as proposed by Du Vigneaud [12]).

DCC/HOBt method [36] to afford Boc-Asp(OBu^t)-Phet-NH_2 with 71% yield. Both protecting groups of the aspartyl residue were cleaved with HCl/CH_3COOH solution and the resulting dipeptide thioamide was coupled with *tert*-butoxycarbonyl-L-tryptophanyl-L-methionine azide (Honzl-Rudiger method [37]). The structure of Boc-Trp-Met-Asp-Phet-NH_2 was checked as being correct by mass spectrometry, amino acid and elemental analysis. The homogeneity of the product was demonstrated by TLC. The details of the synthesis will be published elsewhere (M. Kruszynski, manuscript in preparation).

The values of R_0 were calculated according to eq. 3. The quantum yields of the donor were obtained relative to that of tryptophan in water at 20°C using a value of 0.13 [38], with the corrections introduced into the refractive index of water and propylene glycol and the absorbance of the solutions. Frequency-domain measurements were made using a 2 GHz fluorometer as described previously [39]. The modulated excitation was provided by the harmonic content of a 5 ps train of pulses from an R6G dye laser, which was pumped synchronously by an argon laser. The excitation wavelength of 290 nm was obtained from a Spectra Physics frequency doubler. A multichannel plate photomultiplier was used as the detector (Hamamatsu R1564U). The emission was observed through a 340 nm interference filter, making use of magic-angle polarizer conditions. For all analyses, the uncertainties in phase ($\delta\Phi$) and the modulation (δm) measurements were taken as 0.2 and 0.005, respectively. The analysis of the data in terms of the sum of exponentials was carried out as described previously [40].

4. Results and discussion

The analysis of the absorption spectra (fig. 1) indicates that for the D-A pair the absorption and emission spectra overlap from 300 to 360 nm. This overlap enables energy transfer to occur, resulting in a transfer efficiency of about 60%. It should be noted that the acceptor did not display any detectable emission. The donor quantum yield was found to be 0.37; the overlap integral was $J = 4.5 \times 10^{-16}$ cm^6/M, yielding an R_0 value of 16.9 Å. Such a relatively low value of R_0 is convenient when determining the energy transfer in short peptide chains where the D-A separations are of the order of several ångströms [8,11].

The frequency response of the donor emission at 20 °C is shown in fig. 3. In the presence of energy transfer (D-A pair) the intensity decay is considerably more complex, as is seen from our inability to fit the data (- - - - - -) to a single-exponential decay. The multiexponential analysis of the donor decay of D and D-A is given in table 1. The increase in heterogeneity of the decay in the presence of the thioamide acceptor is evident from the values of χ^2_R from the best single-exponential fits, which increase 6.4-fold for the thioamide peptide. This increase in heterogeneity constitutes evidence in favor of a range of D–A distances. The data were also analyzed using eqs. 4 and 5 to allow for Gaussian distributions of D–A distances (fig. 4). This model results in a good fit to the data

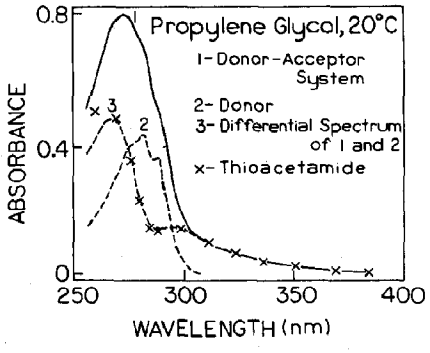


Fig. 1. Absorption spectra of the D-A pair (1), donor (2) and thioacetamide (×) in propylene glycol; (3) difference spectrum of the D-A pair and the donor. The concentration of all samples was 8×10^{-5} M.

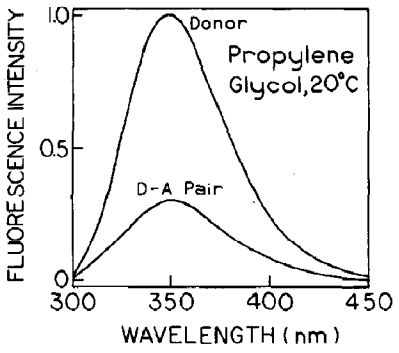


Fig. 2. Emission spectra of the donor and the D-A pair in propylene glycol at 20 °C. All spectra were recorded with excitation at 290 nm.

(table 2, $\chi^2_R = 1.4$), comparable with triple-exponential fits (table 1). The model has only two floating parameters (r and hw), whereas the triple-exponential model contains five adjustable parameters. It is not possible to fit the data to a narrow range of distances. For example, if the hw is held fixed at 1 Å, the data cannot be fitted using eqs. 4 and 5, resulting in $\chi^2_R = 328$ (fig. 4 and table 2). The distance distributions were re-

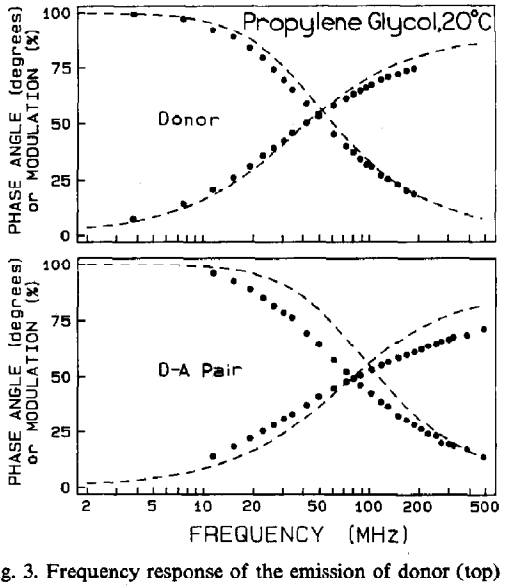


Fig. 3. Frequency response of the emission of donor (top) and the donor-acceptor pair (bottom) (observation at 340 nm). The dashed lines represent the best single-exponential fits to the data.

Table 1

Multiexponential analysis of the donor and donor-acceptor intensity decays at 20 °C in propylene glycol

Compound	τ_i (ns)	α_i	f_i	χ^2_R
Donor	4.56	1	1	138.4
	1.38	0.301	0.096	
	5.59	0.699	0.904	1.5
D-A pair	2.42	1	1	877.4
	0.65	0.553	0.167	
	4.03	0.477	0.833	11.8
	0.33	0.349	0.057	
	1.44	0.342	0.241	
	4.64	0.309	0.702	1.6

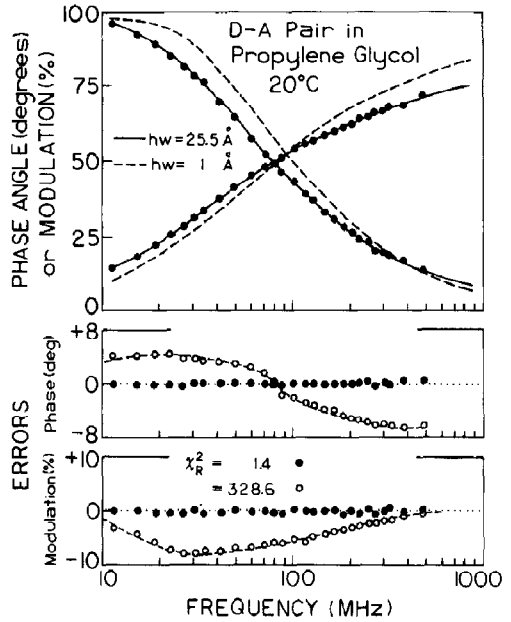


Fig. 4. Distance distribution analysis of the 340 nm emission from the donor-acceptor pair in propylene glycol at 20 °C. The solid line represents the best fit to the data using the decay law of the donor (table 1) and Gaussian distance distribution with $\bar{r}$ = 9.05 Å and hw = 25.5 Å. Also shown is the best fit when the hw is fixed at 1 Å (- - - - -).

Table 2

Distance distribution analysis of the donor-acceptor pair

T (°C)	r (Å) [a]	hw (Å)	χ^2_R
20	9.05 (0.5)	25.5 (0.7)	1.4
20	17.30 (0.4)	⟨1⟩ [b]	328.6

[a] $m = 0$ in eq. 5.

[b] ⟨ ⟩ denotes that hw was held fixed at the value indicated.

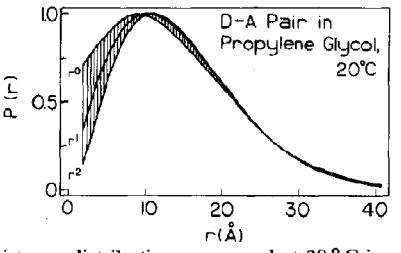


Fig. 5. Distance distributions recovered at 20 °C in propylene glycol using Gaussian distributions multiplied by r^0, r^1 and r^2. The hatched region indicates the maximum range of the $P(r)$ values found using different models.

covered using multipliers of r^0, r^1 and r^2 in eq. 5. Lower powers of $\bar{r}$ are appropriate for shorter chains, whereas r^2 is suitable for an infinite flexible chain [41]. The range of distance distributions recovered with these various models are shown in fig. 5. The value of hw obtained for the thioamide peptide is twice that in the donor-acceptor systems linked with (-CH_2)- bridges [10]. Such a broad range of D–A distance distributions shows that no predominant conformation exists. Despite steric hindrance (such as the phenyl ring) the system can adopt various conformations. The distribution determined is approximate and describes the different solution conformations by means of two parameters, $\bar{r}$ and hw. It is possible that the actual conformational distribution is bimodal, but this is not resolvable with the presently available measurements. It should be noted that the distribution of distances in small peptides can only be mea-

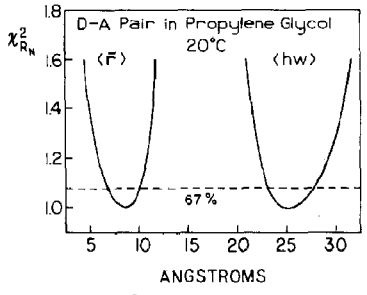


Fig. 6. Dependence of χ^2_R on the values of $\bar{r}$ and hw. These surfaces reflect the experimental uncertainty in the distance distribution. The minimum values of χ^2_R were normalized to unity ($\chi^2_R = 1.0$). The dashed lines indicate the highest values of χ^2_R expected for random noise in repetitive measurements.

sured using a short-range donor-acceptor system, such as the tryptophan-thiocarbonyl system. For example, if a dansyl acceptor were to be used with the present peptide, the transfer efficiency would be greater than 90% ($R_0 = 25.5$ Å for the tryptophan-dansyl system) [10].

Finally, we questioned the uncertainties in $\bar{r}$ and hw. This was accomplished by fitting the data with one parameter fixed while the other was varied to minimize χ^2_R. If the data are adequate for the determination of a parameter, then the value of χ^2_R should vary significantly as the parameter value is altered. If the χ^2_R value is not sensitive to the parameter value, the χ^2_R does not vary. These χ^2_R surfaces are shown in fig. 6. The horizontal dashed lines indicate the value expected 67% of the time for random errors. For the D-A pair the uncertainties in both $\bar{r}$ and hw are relatively small, near 2 and 3 Å, respectively.

In conclusion, frequency-domain fluorometry in combination with the data on tryptophan to thioamide energy transfer allows the resolution of distance distributions in small peptides. Such data can be used for comparison of the measured conformational distribution with that predicted from theoretical potential functions for amino acid residue conformations.

Acknowledgements

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