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ARTICLE

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Conformational analysis of galanin using end to end distance distribution observed by Förster resonance energy transfer

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Abstract The structural dynamics of the flexible neuropeptide galanin in solution were studied by Förster resonance energy transfer measurements at different temperatures by time-resolved fluorescence spectroscopy to determine its conformational heterogeneity. Endogenous tryptophan at position 2 acted as the fluorescent donor and the non fluorescent acceptor dinitrophenyl or the fluorescent acceptor dansyl were selectively attached to lysine 25 in porcine galanin. The coexistence of different structures of the neuropeptide galanin in trifluoroethanol solution was revealed by the model independent analysis of the distribution of relaxation times from the time-resolved resonance energy transfer data. Multiple conformational states are reflected by distinct end-to-end distance populations. The conformations differ in mean donor-acceptor distance by about 15 Å, and are consistent with the extended and folded backbone conformations of two α-helical regions separated by a flexible hinge. The effect that the labelling of galanin has on binding to the receptor was also evaluated. DNP-galanin showed the same high affinity to galanin receptors as unlabelled galanin, whereas DNS-galanin had significantly reduced affinity.

Key words Neuropeptide · Conformational dynamics · Time-resolved fluorescence spectroscopy · Binding studies

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Abbreviations BSA Bovine serum albumin · CD Circular dichroism · DNS Dansyl · DCM Dichloromethane · DIEA Diisopropylethylamine · DMF Dimethylformamide · DMSO Dimethylsulphoxide · DNFB 2,4-dinitrofluorobenzene · DNP Dinitrophenyl · FRET Förster resonance energy transfer · FT-IR Fourier transformed infrared · HOBt hydroxybenzotriazole · HEPES N-[2-hydroxiethyl]-piperazine-N′-[2-ethanesulfonic acid] · Lys Lysine · MBHA 4-methyl-benzylhydryl amine · NMP N-methyl pyrrolidone · NMR Nuclear magnetic resonance · NOE Nuclear Overhauser effect · TFE Trifluoroethanol · Trp Tryptophan · TFA Trifluoroacetic acid

Introduction

The biological function of proteins often involves conformational changes. The extent of these changes and the mechanism of their induction differs, depending on the complexity and type of protein. In allosteric systems, for example, conformational changes are triggered by the interaction of the protein with its effectors in defined binding sites. A particular group of effectors consists of small proteins: biologically active neuropeptides. These typically exhibit random coil conformation in water, but are thought to adopt a defined structure upon binding to the receptors. In these molecules, the conformational propensity is a diffuse property of the whole molecule, which behaves like a flexible structureless polymer attaining a defined specific structure only in very particular conditions. The knowledge of the formation of this structure and its dynamic properties is a prerequisite for the understanding of how the neuropeptide interacts with its receptor and especially for the development of a rational design for analogues of the active neuropeptides. The conformational dynamics make structural studies difficult, if not impossible, with traditional approaches such as X-ray crystallography or NMR.

Galanin is an example of a small peptide in which conformational flexibility is likely to be important for its

biological function. It is a peptide of 29–30 amino acid residues and was first isolated from the small intestine of the pig (Tatemoto et al. 1983). It has also been found in other tissues such as brain, spinal cord and in various parts of the peripheral nervous system in different species (Rökaeus 1987; Bartfai et al. 1993). Galanin exhibits several biological properties (Bartfai et al. 1993). It modulates the physiological effects of growth hormone, prolactin, dopamine, insulin, somatostatin and glucagon, can function as a neurotransmitter (Melander et al. 1986) and can act as an antagonist of substance P in pain relief (Wiesenfeld-Hallin et al. 1992).

The structure of galanin in solution has been examined by NMR, CD and FT-IR spectroscopy, studies which have shown that this peptide has no defined secondary structure in aqueous solution, but forms an α-helical structure in TFE (Rigler et al. 1991; Wennerberg et al. 1990). The tertiary structure has been determined by 2D-^{1}H-NMR spectroscopy (Wennerberg et al. 1990). Galanin in TFE consists of α-helical regions separated by a flexible kink due to a proline residue at position 13. Computer simulations of the molecular dynamics of galanin in water and in TFE have also been carried out in order to investigate the influence of different solvents on the conformation and stability of the structure (De Loof et al. 1992).

During the analysis of the NMR data, several structures consistent with the distance constraints derived from NOE data could be generated. They show a wide diversity, especially regarding the overall structure of the molecule (Wennerberg et al. 1994b), suggesting that different structures may coexist in a dynamic equilibrium instead of one defined structure, at least on the NMR time-scale. Analysis of the distribution of these structures based only on NOE data from NMR spectroscopy is difficult because of the lack of the long distance constraints necessary for the unambiguous definition of an elongated structure. We have therefore turned to Försters resonant energy transfer (FRET) measurements as a complementary technique capable of providing data about end-to-end distances for galanin in solution.

Porcine galanin contains an endogenous fluorophore close to the N-terminus, Trp2, which was used as the fluorescent donor [Gly-Trp^2-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro^{13}-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-His-Asp-Lys^{25}-Tyr-Gly-Leu-Ala-NH_2]. Two different labels attached to the ε-amino group of Lys^{25} were used as excitation energy acceptors, DNS or DNP. The biological relevance of the introduced chemical modification has been controlled by binding studies.

A relaxation-time distribution analysis of the experimental results has been performed to obtain the distribution function of these distances instead of one averaged value for the investigated system. This approach enables us to investigate the coexistence of different subpopulations and the equilibrium between them under the influence of different factors and gives structural information complementary to the 2D-NMR spectroscopy. The combination of these two techniques may create new possibilities for the investigation of the dynamics of structure formation during protein folding, as well as its functional conformational changes. Together their increased experimental accuracy and resolution can yield insights into real protein structural dynamics, extending the static picture obtained with atomic resolution by crystallographic measurements whose intrinsic limitations may thus be circumvented.

Materials and methods

Peptide synthesis

The peptide backbone of porcine Lys25(ε-Fmoc)-galanin(1-29) amide was assembled in a stepwise manner on a solid support using an Applied Biosystems Model 431A Peptide Synthesizer and the standard NMP/HOBt Solvent-Activation strategy on a 0.1 mmol scale. tert-Boc-Amino-acids and tert-Boc-Lys(ε-Fmoc) were coupled to 4-methylbenzylhydryl amine resin (Bachem Feinchemikalien AG, Bubendorf, Switzerland) as hydroxybenzotriazole esters.

An individual cycle for amino acid included deprotection of the tert-Boc-group with 50% (v/v) trifluoroacetic acid in CH_2Cl_2 for 19 min, and acylation with 10-fold excess (compared to the amount of amino groups on a resin) of the protected amino acids in a mixture of 15% dimethylsulphoxide (v/v) in N-methyl pyrrolidone for 35 min. Between each operation, several extensive washings were performed with DCM, diisopropylethylamine and NMP. After each coupling acetylation (capping) was carried out using 10% (v/v) acetic anhydride and 5% DIEA (v/v) in NMP for 5 min.

The 2,4-dinitrofluorobenzene (Sanger's reagent, Sigma) was coupled to the peptide-resin deprotected from Lys25 (εNH-Fmoc)-group as follows. To deprotected and base-washed peptide-resin in DCM/dimethylformamide, 1:1, 2 equivalents of DNFB and 4 equivalents of DIEA were added and the mixture was stirred for 1 h at room temperature. The washings were performed with DCM and DMF and the coupling of DNFB was confirmed by a ninhydrin test. In order to obtain DNS-labelled analogues of porcine galanin, the dansyl group was coupled to the amino group in excess dansyl chloride (Sigma, USA) in the same manner as described above.

Deprotection of the side-chain protective groups by "low TFMSA" method and cleavage of the peptides from the resin by making use of liquid HF have been described earlier (Land et al. 1991a). Purification of all the cleaved peptides was carried out on an LKB HPLC apparatus (SYSTEM PREP 50) using Polygosil 60-7 C18 reversed-phase column (1.0 cm×25.0 cm). The crude peptide fragments were separated at a flow rate of 2.0 ml/min, using a linear gradient in a system of 0.1% (v/v) trifluoroacetic acid/H_2O (solvent A) and 0.1% (v/v) trifluoroacetic acid/CH_3CN (solvent B). The gradient ranged over 20–60% (v/v) of solvent B in 40 min. Purity of the individual peptides was checked by analytical Nucleosil 120-3 C18 reversed-phase HPLC column (0.4 cm×10.0 cm) and de-

termined to be 99% or higher. Molecular weights of the peptides were determined using Plasma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, values corresponding to those calculated being obtained.

Binding studies

Membrane preparations from rat cerebral cortex and receptor binding analyses carried out by a filtration technique as described earlier (Land et al. 1991b), using 10 mM HEPES-buffered (pH 7.4) KREBS-RINGER solution containing 1 mg/ml bacitracin and 1 mg/ml BSA. The mixtures were incubated for 30 min at 37 °C using ^{125}I-galanin (0.2 nM) as a radioligand employed at a subsaturating concentration and different concentrations of the peptides to be tested. Specific binding was defined as that portion of the total binding which could be reserved by excess of unlabelled galanin (1 μM) or other ligands (1 μM). Fitting of the binding equation to the experimental data was carried out by means of a non-linear least squares method using the program Kaleidagraph.

Fluorescence measurements

Time-resolved fluorescence measurements were performed with a time-correlated single-photon-counting laser spectrometer (Rigler et al. 1985). Tryptophan was excited at wavelength of 300 nm, and the emission was measured through a set of two cut-off filters, WG 345 and UG11, creating a spectral window between 340 and 400 nm. Fluorescence of the dansyl group attached to Lys25 was measured using excitation at 306 nm and monitored through a cut-off filter KV500. The fluorescence intensity decay and the anistropy decay were measured with magic and perpendicular orientations of the analysing polarisers, respectively (Rigler et al. 1985).

The fluorescence decay of the donor becomes faster in the presence of the energy-accepting chromophore due to the additional competing radiationless transfer rate. With the assumption that all conformational substates *i* related to the presence of different decay rates of the donor emission are characterised by the same FRET parameters, the fluorescence decay in the presence of an acceptor is described with an additional term, the energy transfer rate $k_T(r)$:

$$I(t) = \int P(r) \sum_i a_i \exp\{-(k_D^i + k_T^i(r))\, t\}\, dr \qquad (1)$$

where $P(r)$ is the probability density for the different donor-acceptor distances r occurring in the different macromolecular conformations.

The analysis of the experimental data in terms of the distribution of energy transfer rates involves two pairwise (donor alone and donor-acceptor) steps. First a numerical analysis with a non-linear parametrisation procedure (Marquardt 1963) using convolution of the model function with the measured excitation pulse was applied for the donor fluorescence with the assumption that the fluorescence decay consisted of one or several exponential components. The number of exponentials was selected according to the quality of the fit improvement as judged by the reduced χ^2 value.

In the second step, the donor decay of the donor-acceptor pair in the peptide was analysed. For this purpose we analysed the donor-acceptor pair distance distribution by a procedure which yields model free distribution function in contrast to the often used explicit analytical distribution functions (Lakowicz et al. 1988, 1990; Rice et al. 1991; Wu et al. 1991). The distribution is obtained from a linear contribution of discrete elements using a constrained regularization as provided by the CONTIN program described extensively elsewhere (Provencher 1982). No assumption of the number of parameters necessary for a statistically acceptable quality of the fit is required prior to the evaluation. The intensity of the donor fluorescence following the excitation is:

$$I(t) = \sum_{r_{\min}}^{r_{\max}} P(r)\, K(r,t) \qquad (2)$$

where

$$\mathrm{K(r,t)} = \sum_{\mathrm{j=1}}^{\mathrm{N}} \mathrm{a_j} \int_0^{\mathrm{t}} \mathrm{L(t-t')} \exp[-\{1+(\mathrm{R_0/r})^6\}\, \mathrm{t'}/\tau_{\mathrm{j}}]\, \mathrm{dt'} \qquad (3)$$

with a_j, τ_j – the amplitudes and decay times for galanin without energy transfer, respectively, $L(t)$ is the response (lamp) function.

In the model function, the discrete distribution function $P(r)$ is estimated by the (non-negatively) constrained regularization method CONTIN with a spacing of 1 Å between points. The limits $r_{min} = R_0/4$ and $r_{max} = 2.5\ R_0$ are well beyond the experimentally accessible values for r and therefore the integration interval includes all observable values.

It turned out that spurious amounts of Fmoc could still be detected in the sample after deprotection, labelling and extensive purification as a long lived fluorescent component. It turned our that it is possible to perform the evaluation with CONTIN even in the presence of fluorophores which are not involved in FRET, but overlapping with the emission spectrum of the donor if they are characterized by a distinctly different fluorescence lifetime. With the contaminant correction the model becomes:

$$I(t) = \sum_{R_{\min}}^{R_{\max}} P(r)\, K(r,t)\, dr + \gamma \int_0^t L(t-t') \cdot \exp(-t'/\tau_c)\, dt', \qquad (2\,a)$$

where γ is the fractional contribution of the contaminant (estimated by CONTIN) characterised by decay time τ_c.

The rate of the energy transfer k_T between donor (D) and acceptor (A) is described (Förster 1948) by:

$$k_T = (1/\tau_D)\, (R_0/r)^6 \qquad (4)$$

where r is the distance between donor and acceptor and R_0 is the characteristic (Förster) transfer distance defined by

spectral properties of the donor and acceptor and the surrounding medium:

$$R_0^6 = \frac{9000 \ln(10) \kappa^2 \Phi_D}{128 \pi^5 n^4 N_A} J_{\tilde{\nu}} \quad (5)$$

Φ_D is the quantum yield of the donor fluorescence in the absence of the acceptor, n is the refractive index of the surrounding medium, N_A is Avogadro's number, and $J_{\tilde{\nu}}$ is the spectral overlap integral of emission and absorption bands of donor and acceptor:

$$J_{\tilde{\nu}} = \int_{\tilde{\nu}} F_D(\tilde{\nu}) \varepsilon(\tilde{\nu}) \frac{d\tilde{\nu}}{\tilde{\nu}^4} \approx \sum_i F_D(\tilde{\nu}_i) \varepsilon(\tilde{\nu}_i) \frac{\Delta\tilde{\nu}_i}{\tilde{\nu}_i^4} \quad (6)$$

where $\tilde{\nu}$ is the wavenumber, $F_D(\tilde{\nu})$ is the normalised fluorescence intensity per unit wavenumber, $\varepsilon_A(\tilde{\nu})$ is the acceptor molar absorptivity at the wavenumber $\tilde{\nu}$. The molar absorptivity of DNP is 17 000 $M^{-1} cm^{-1}$ at 349 nm and the molar absorptivity of DNS is 4600 $M^{-1} cm^{-1}$ at 336 nm (Molecular Probes Inc., USA). The refractive index of TFE is 1.290 (Weast & Astle 1978).

κ is the orientation factor which gives the dependence of the interaction between two electric dipoles on their relative angular orientation (Förster 1948):

$$\kappa = (\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A) \quad (7)$$

where θ_{DA} is the angle between the donor (D) and acceptor (A) transition moments, θ_D, θ_A are the angles between the separation vector R and donor D, and R and acceptor A transition moments, respectively. It is evident from Eq. (6) that in a system with transition dipole moments rigidly positioned, the energy transfer efficiency is strongly dependent on the relative orientation of the two chromophores. A single defined value of the orientation factor κ is valid only if the experimental system consists of D-A pairs with the same fixed orientations or is characterized by D-A pairs having some extent of dynamic average about a fixed mean orientation (Dale et al. 1979).

For the system with fluorophores showing some orientational freedom, dynamic averaging of the orientations takes place. Several models of the relative orientations of the donor emission and acceptor absorption dipoles have been theoretically analysed for systems with rapid restricted motions of luminophores (Dale and Eisinger 1974) and average values for the orientation factor κ for any relative orientation have been calculated as a function of the degree of restricted motion (Dale et al. 1979). The degree of restricted motion of donor or acceptor may be obtained by the use of the information available from polarization measurements (Kinosita et al. 1977; Lipari and Szabo 1980). For wobbling-in-a-cone model with the assumption that the absorption or emission transition moments are oriented along the wobbling axis of the chromophore:

$$r(t)/r_0 = (1-S^2) \exp[-t(\phi_p^{-1} + \phi_{eff}^{-1})] + S^2 \exp(-t/\phi_p) \quad (8)$$

where ϕ_p, ϕ_{eff} are rotatioal correlation times of the protein and wobbling diffusion, respectively (Lipari and Szabo 1980). The order parameter *S*, and the angle of the wobbling cone θ are related by:

$$S^2 = r_2/r_0 = [(1/2)(\cos\theta_0)(1+\cos\theta_0)]^2 \quad (9)$$

(Kinosita et al. 1977; Lipari and Szabo 1980).

Hence, the extreme values of the orientation factor for different angular orientations of the chromophores can be experimentally evaluated from the *S* obtained for the donor and the acceptor, (Dale et al. 1979). The effective orientation parameter may represent a statistically averaged value, for instance when the structure exhibits a distribution of orientations of subunits.

Results

Displacement of ^{125}I-galanin by DNS- and DNP-galanin

In order to control the biological relevance of the introduced chemical modifications, the binding studies were performed in rat hypothalamic membranes. Both, DNA- and DNP-galanin displaced ^{125}I-galanin from its complex with hypothalamic galanin receptors with high affinity. However, the binding affinity for DNP-galanin was considerably higher, K_D=0.8 nM and very similar to the affinity of the native galanin, K_D=0.9 nM (Land et al. 1991a), compared with the affinity of DNS-galanin (K_D=32 nM).

Determination of R_0 for labelled galanin

The Förster's critical distance, R_0, for donor-acceptor pairs Trp-DNS and Trp-DNP were determined according to Eq. (5). The spectral overlap integral, $J_{\tilde{\nu}}$, divided by Avogadro's number, N_A, was estimated to be $3.3 \pm 0.2 \times 10^{-38}$ cm^6 for Trp-DNP and $9.6 \pm 0.2 \times 10^{-39}$ cm^6 for Trp-DNS. $J_{\tilde{\nu}}$ was calculated from the normalized fluorescence spectrum of Trp2 in galanin and the absorption spectrum of dinitrophenyl/dansyl diluted in TFE according to Eq. (6), see Fig. 1. The quantum yield of the donor (Trp) was calculated using as reference the value of 0.14 for tryptophan in water (Chen, 1967).

R_0 was calculated for TFE as solvent at two temperatures +20 °C and −20 °C. For Trp-DNP pair this gave R_0=26.4 and 30.4 Å, for the Trp-DNS pair R_0=21.5 and 24.7 Å, respectively. The R_0 for the low temperature conditions (−20 °C) was calculated assuming the proportionality between the quantum yield and average lifetime of the fluorescence of the donor.

Fluorescence decay of tryptophan

The fluorescence decay of Trp2 in galanin is presented in Fig. 2. The analysis of the decay of tryptophan fluorescence in single tryptophan proteins usually requires a multiexponential function to describe the time-dependence of fluorescence intensity, related to different micro envi-

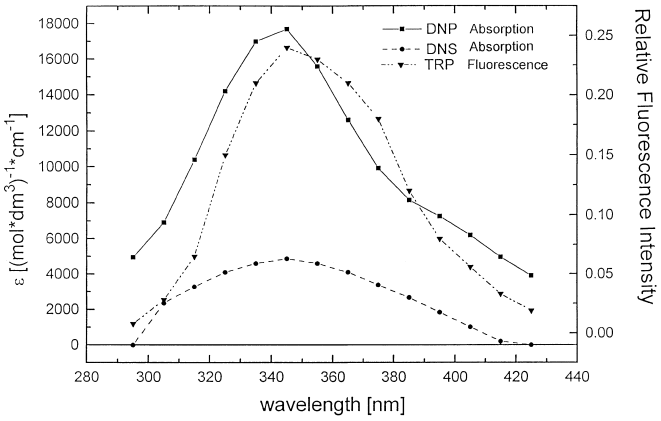

Fig. 1 The spectral overlap between the donor-acceptor pairs used in this study. The fluorescence spectrum of Trp and the absorption spectra of DNS and DNP, illustrating the spectral overlap of Trp emission with the DNS respectively the DNP excitation spectrum. Fluorescence spectra for Trp, *open triangles* and *dotted line*; absorption spectrum for DNS *filled circles* and *dashed line*; for DNP *filled squares* and *solid line*

ronments sensed by the tryptophan residue (Beechem and Brand 1985). The analysis of fluorescence of Trp2 in galanin revealed the presence of a minimum of four exponential components. The fluorescence lifetimes and corresponding pre-exponential coefficients are given in Table 1.

Distance distribution analysis

The fluorescence decay of Trp2 in the presence of either DNP or DNS linked to Lys at position 25 becomes faster than in the absence of acceptor owing to resonance energy transfer. The fluorescence intensity decay of acceptor (DNS or DNP) labelled galanin could be analysed in several ways, including the multiexponential model and lifetime distribution model (CONTIN). The multiexponential free parameter analysis of the donor decay shows that its lifetimes has changed, but the preexponential factors are in the same range as for nonlabeled galanin. A minor additional fluorescent component of the lifetime longer than any component of tryptophan, but close to the fluorescence of the Fmoc is present in the recorded emission.

The analysis of the distribution of the transfer rate term, Eq. (1), provides an appropriate decay model as compared to a discrete exponential components model. This analysis includes the fluorescence decay parameters of unlabelled galanin, contamination component and minimization with the CONTIN routine and gives a solution with χ^2 in the range of 1.08. These results of the measurements of the distance between Trp2 and DNP-Lys25 shows the existence of two D-A distance populations with their maxima separated by approximately 15 Å. At +20 °C the two

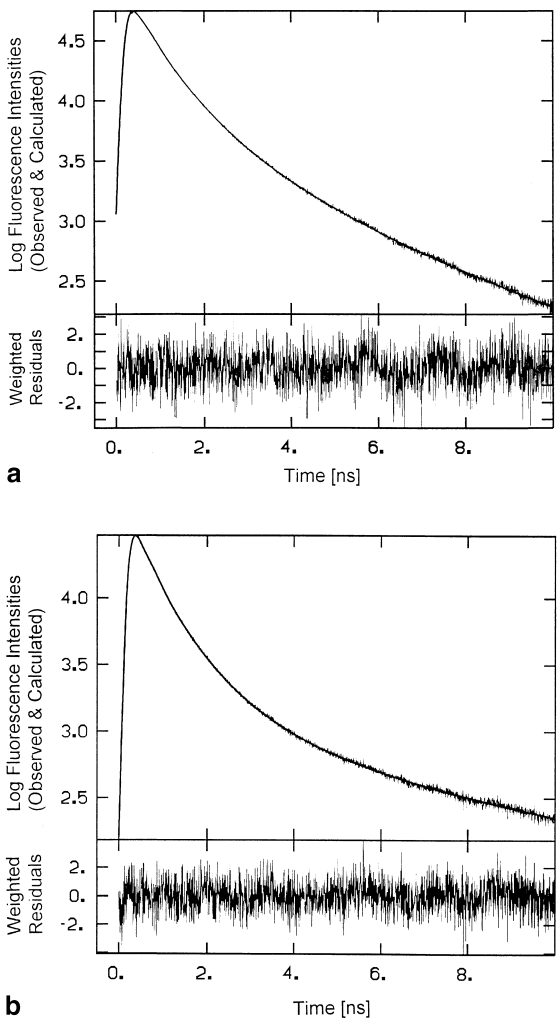

Fig. 2 Fluorescence intensity decays: **a** Trp2 of galanin in TFE at +20 °C, **b** Trp2 of galanin in TFE with energy transfer acceptor DNP-Lys25. **c** Trp2 to DNP-Lys25 and to DNS-Lys25 distance distributions in galanin in TFE obtained by the analysis of the energy transfer experiments with CONTIN. Distance distribution for Trp and DNP at +20 °C, *filled squares* and *solid line*. Distance distribution for donor-acceptor pair Trp and DNP at −20 °C, *open squares, dotted line*. Trp and DNS at −22 °C, *open circles* and *dashed line*

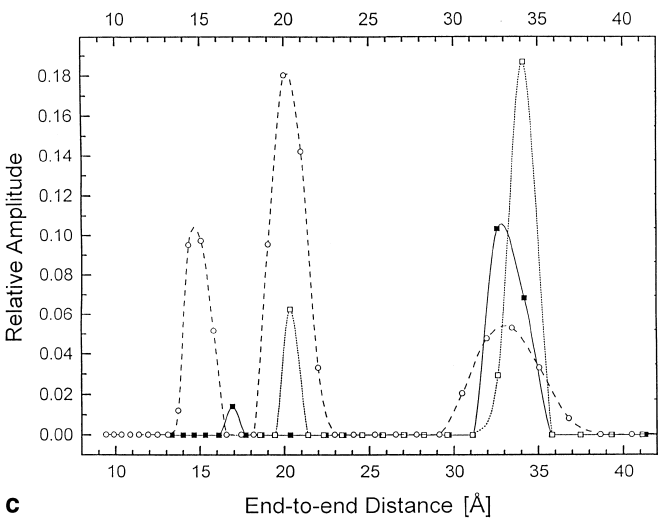

Table 1 Lifetimes and amplitudes of the fluorescence of Trp2 in galanin measured at different temperatures. τ_m denotes the mean fluorescence lifetime

Lifetime (amplitude)	TFE −20 °C [ns]		TFE +20 °C [ns]		Water +20 °C [ns]	
τ_1 (a_1)	0.20 ± 0.05	(0.26 ± 0.05)	0.24 ± 0.06	(0.41 ± 0.05)	0.24 ± 0.2	(0.23 ± 0.1)
τ_2 (a_2)	1.2 ± 0.1	(0.39 ± 0.1)	0.8 ± 0.1	(0.48 ± 0.1)	1.1 ± 0.2	(0.58 ± 0.1)
τ_3 (a_3)	2.6 ± 0.2	(0.26 ± 0.05)	2.2 ± 0.3	(0.09 ± 0.05)	2.2 ± 0.4	(0.17 ± 0.05)
τ_4 (a_4)	5.7 ± 0.5	(0.09 ± 0.05)	6.2 ± 0.5	(0.005 ± 0.03)	6.2 ± 0.8	(0.01 ± 0.01)
τ_m	1.69		0.72		1.11	
χ^2	1.18		1.22		1.07	

Table 2 Rotational correlation times (ϕ_i), partial anisotropies (β_i), order parameters (S) and wobbling cone angles (Θ_0) measured for Trp2 and DNS-group attached to Lys25 in porcine galanin

	Temperature [C]	ϕ_1 [ps]	ϕ_2 [ns]	β_1	β_2	S	Θ_0 [°]
Trp2	−20 °	50 ± 20	4.9 ± 0.3	0.16 ± 0.01	0.17 ± 0.01	0.72	34.5
Trp2	+20 °	35 ± 20	2.2 ± 0.3	0.17 ± 0.01	0.16 ± 0.01	0.69	36
DNS-Lys25	−20 °	440 ± 50	4.7 ± 0.3	0.08 ± 0.01	0.09 ± 0.01	0.73	34.0
DNS-Lys25	+20 °	210 ± 50	2.1 ± 0.3	0.12 ± 0.01	0.11 ± 0.01	0.69	36

peaks correspond to distances of 33 Å and 17 Å with normalized populations of 0.92 and 0.08 of the total, respectively. At −20 °C, the recovered distances have their distribution maxima at 33 Å and 20 Å with fractional populations of 0.87 and 0.13, respectively (Fig. 2c).

The results of the experiments with DNS labelled galanin analysed with an unregularised routine have been presented elsewhere (Kulinski et al. 1993). A three-modal distance distribution characterizing the separation between donor and acceptor was found at −20 °C for the system labelled with DNS at Lys25 with maxima at 16 Å, 21 Å and 34 Å contributing to the transfer rate in non-equal fractions, corresponding to 27, 52 and 21 percent of the whole population (reduced $\chi^2 = 1.07$). The distance distribution between the two chromophores within the galanin molecule obtained with the regularized routine now employed is presented in Fig. 2c. This gave a two-peaked solution, with only slightly worse fit (reduced $\chi^2 = 1.085$). The regularized solution evidently merges the two peaks at 16 and 21 Å into one, placed around 20 Å, which corresponds well with the weighted mean value of the two previously identified peaks in this region.

Rotational correlation times

The results of the fluorescence anisotropy decay measurements for Trp(2) and DNS(Lys25) in galanin are presented in Fig. 3 and the decay parameters obtained are summarized in Table 2. The measurements were made at +20 °C, and also at −20 °C, where additional stabilization of the structure may be expected. Excitation of Trp at 300 nm leads to the high initial anisotropy because then it is almost exclusively the emitting ^1L_a transition that is excited (Munro et al. 1979). Rotational correlation times were evaluated from the ratio of the fluorescence decay measured with the orientation of the analysing polarizer perpendicular to the polarisation of the exciting beam to that measured with the orientation at the magic angle I_{perp}/I_{magic} (Ehrenberg and Rigler 1976; Ruggiero et al. 1990).

The anisotropy decay was found to be adequately described by two correlation times. The short component of around 35−50 ps can be ascribed to local tryptophan motions, the longer one to the overall tumbling of the whole molecule. The temperature dependence of both of these components is due to the change of viscosity of the solvent with temperature. The long correlation time ascribed to the overall rotation of the whole molecule is directly related to its volume and should give an indication of possible aggregation of galanin in concentrated solutions. The fluorescence anisotropy of galanin in TFE was measured over a wide range of concentrations, from 8×10^{-3} M as used for NMR measurements (Wennerberg et al. 1990) to 10^{-6} M. There was no appreciable change in either rotational correlation time, indicating that there is no noticeable aggregation at the concentrations of galanin used for NMR experiments (Table 3).

The viscosity dependence of the short correlation time suggests that the fluorescent residue shows free rotations within a restricted site rather than reorientation ("jump") kinetics between a number of sites in which local rotation relaxation is absent, or between several different confor-

Table 3 Fluorescence anisotropy of Trp2 in galanin measured in TFE at different concentrations at +20 °C: ϕ_1 – rotational correlation times, β_i – partial anisotropies

Concentration [mM]	ϕ_1 [ns]	β_1	ϕ_2 [ns]	β_2
8	0.03 ± 0.02	0.18 ± 0.01	2.3 ± 0.02	0.16 ± 0.01
0.8	0.03 ± 0.02	0.19 ± 0.01	2.1 ± 0.02	0.16 ± 0.01
0.05	0.03 ± 0.02	0.18 ± 0.01	2.2 ± 0.02	0.15 ± 0.01
0.01	0.03 ± 0.03	0.20 ± 0.02	2.1 ± 0.03	0.16 ± 0.2

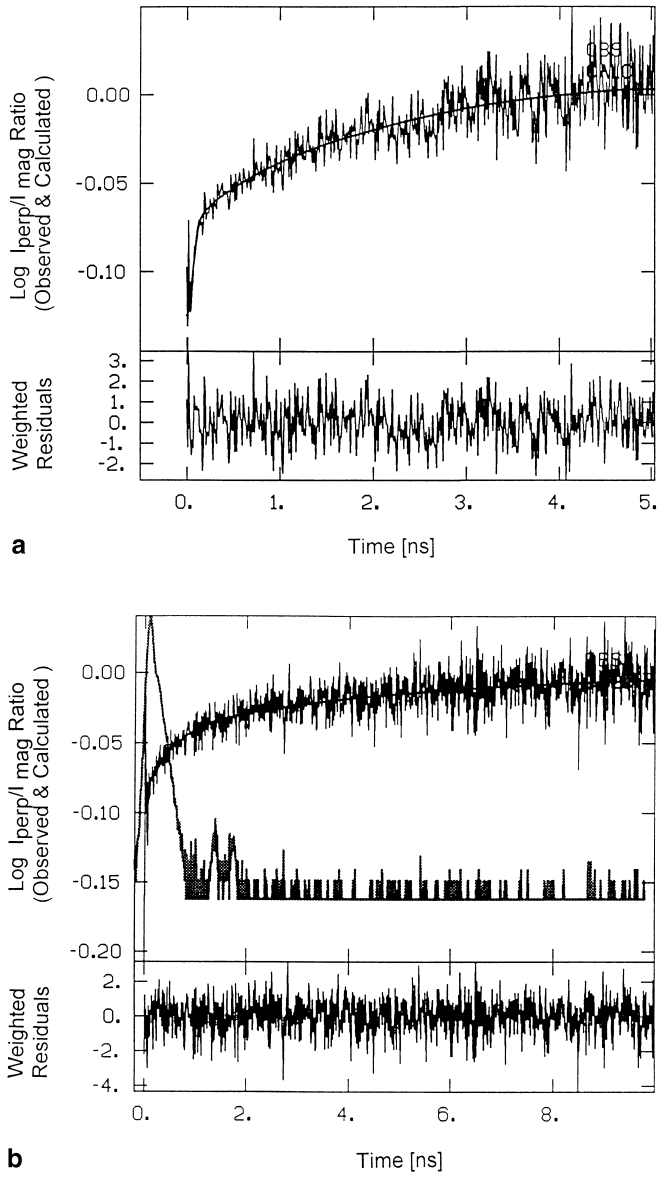

Fig. 3 Fluorescence anisotropy decay of galanin: **a** Trp2 in TFE at +20 °C. **b** DNS-Lys25 in TFE excited directly at 308 nm at +20 °C. The data are presented as a ratio of the experimental Iperp(t) to Imagic(t) (*upper panels:* noisy main curve with fit and excitation function; *lower panel:* weighted residuals for the fit)

mations with respect to the whole molecule (Gottlieb and Wahl 1963).

Orientation dependence of energy transfer rate

There are two unknowns involved in the energy transfer process: the distance between the donor and acceptor chromophores and the relative orientation of their transition moments. The rate of Förster resonance energy transfer is orientation dependent. For invariant relative orientations, the orientation factor, κ^2, can vary from 0 to 4, leading to considerable uncertainty in the estimation of distances. Fluorescence anisotropy measurements can reduce the degree of uncertainty, particularly if either or both donor and acceptor exhibit some rapid reorientation relaxation (Dale et al. 1979). When a non-fluorescent energy acceptor is used, the direct experimental determination of the extent of such dynamic orientational averaging is more difficult. In this case the limits for the spread in possible values of the orientation factor were estimated on the assumption that the orientation of the acceptor is fixed.

Table 4 Extreme orientation parameters κ^2 (κ^2_{max}, κ^2_{min}) and corresponding critical Förster distances R_{0max}, R_{0min} calculated for Trp2 and DNS-Lys25 and for Trp2 and DNP-lys25 in pig galanin (orientation of transition dipoles and separation vector parallel-in-line and mutually perpendicular, respectively). R_0 is calculated with $\kappa^2 = 2/3$, according to Dale and Eisinger (1974)

Temperature	DNS −20 °C	DNS +20 °C	DNP +20 °C
κ^2_{max}	2.68	2.55	2.96
κ^2_{max}	0.186	0.20	0.13
$(\kappa^2_{max})^{1/6}$	1.18	1.17	1.20
$(\kappa^2_{min})^{1/6}$	0.76	0.77	0.71
R_{0max}/R_0	1.26	1.25	1.28
R_{0min}/R_0	0.81	0.82	0.76

Extreme orientation factors for the system Trp2-DNS-Lys25 were calculated using the experimental fluorescence anisotropy decay data and are presented in Table 4. These two limiting cases correspond to the most favoured and the most unfavoured orientation of transition dipoles and separation vector, being parallel-in-line and mutually perpendicular, respectively (Dale et al. 1979).

The spread of the value of the orientation factor between the maximal and minimal values results finally on the calculation of the Förster characteristic distance R_0, and hence on the determination of r, in reasonably limited way: giving about ±23% at −20 °C, ±22% at +20 °C uncertainty (Table 2).

Discussion

Solution structure of galanin

Studies of the structure of galanin in solution by NMR, CD, and IR spectroscopy have shown that this peptide does not have a defined secondary structure in aqueous solution, but forms a highly α-helical structure in trifluoroethanol. The analysis of the tertiary structure of galanin as determined by 2D-^{1}H-NMR spectroscopy (Wennerberg et al. 1990) shows that, under these conditions, galanin consists of two α-helical regions separated by a flexible kink due to a proline residue at position 13. A wide diversity of model structures could be generated by distance geometry calculations within the distance constraints derived from NOE data (Wennerberg 1994). This suggests that different conformations, mainly with regard to the

overall structure of the molecule, coexist in TFE in a dynamic equilibrium instead of a single defined structure. A trivial explanation for this diversity may reside in the lack of long distance constraints necessary for the unambiguous definition of an elongated structure. The appropriate interpretation of the NMR spectrum also requires the information that the peptide exists in monomeric form under experimental conditions, and the observed NOE signals are not due to the intermolecular interactions of aggregated galanin. The application of time-resolved fluorescence spectroscopy presented here is capable of providing the supplementary information about the long distance constraints and monodispercity required: time-resolved fluorescence depolarisation and resonance energy transfer measurements supply this data.

Rotation motion, internal motion

Observation of the time dependence of fluorescence anisotropy gives direct insight into molecular mobility. Substantial independent mobility of both Trp2 and DNS-Lys25 fluorophores has been established for galanin, superimposed on the overall rotations of the whole molecule. The long correlation time ascribed to the overall rotations of the whole molecule depends on the volume (molecular mass) of the molecule, its 3-dimensional structure and segmental flexibility and on the viscosity of the solvent. However, it is a characteristic parameter for specific conformations of the molecule and changes in its parameters are good indicators of structural changes, allowing intermolecular interactions and associations (e.g., self aggregation) easily to be monitored. Dependence of the rotational correlation time on the concentration of the substance in dilute solution is indicative of aggregation. Over a wide range of concentrations no appreciable change in rotational correlation time was observed (Table 2), indicating the absence of aggregation at the concentration of galanin used in the previous NMR experiments (Wennerberg et al. 1990; Wennerberg 1994). The NOE signals observed there are thus due exclusively to intramolecular magnetization exchange.

Knowledge of the extent of the mobility of the donor and acceptor is necessary for a proper evaluation of the orientation parameter in characterizing resonance energy transfer. Both chromophores exhibit a rather large freedom of the mobility with respect to the whole molecule, which leads to essentially dynamic reorientational depolarisation. Determination of depolarisation factors for the two chromophores allowed the evaluation of extreme orientation parameters κ^2 for the configurations with the highest and the lowest possible rates of energy transfer (Dale et al. 1979). Additionally, the ratio of the pre-exponential factors found for donor decay components with free-parameter multi-exponential analysis do not differ substantially from that of non-labeled galanin. This suggests that all the donor lifetime components behave in the same way with respect to transfer to the donor and the model assuming simple addition of the competing transfer rate to each component is allowed.

Distance distribution

The apparent distance distribution from time-resolved resonance energy transfer contains different contributions: the true distance distribution and the orientational distribution. The extreme orientation factors calculated for the system Trp2-DNS-Lys25 (Table 3) lead to an uncertainty in the derived distances R_0 of about ± 23% at and ± 22% at + 20 °C. Since no information on the local reorientational mobility of the non-fluorescent DNP-Lys25 was available, the extreme values of the orientation factor when DNP was the acceptor were estimated for the most unfavorable situation, i.e., assuming that the dinitrophenyl ring is rigidly bound to the molecular frame (Table 4). The diversity of real donor–acceptor distances should be smaller than these estimates.

The precision of determination of distance by energy transfer measurements depends also on the relative value of the apparent distance, r, with respect to the Förster distance, R_0. The separation distance determined for the extended form of galanin should be determined quite accurately, since it is comparable with the Förster distance, R_0, for both donor–acceptor pairs used. The folded form is determined with less accuracy owing to the large quenching of the donor at shorter donor–acceptor distance and the dominant presence of the extended form in the system, and the shape of the peak related to the folded form should be regarded as a rougher estimate than that of the extended one.

An analytical model which would properly describe the distribution of distances in the investigated system is difficult to specify. Theoretical modelling of polymers has so far yielded a defined expression for the distance distribution only for completely flexible structures, such as randomly coiled peptides in solution (Flory 1969). For semi-rigid molecules, it is more difficult to obtain easily handled expressions describing the distance distributions of elements of the structure. Relatively simply parameterized models such as Gaussian or Lorentzian distribution provide one reasonable way aproach the problem. It has been established in the analysis of both real and simulated data, that the average distance of a distribution can be determined quite precisely but the shape of the distribution is determined with less accuracy (Wu and Brand 1992).

The Trp2 to DNP-Lys25 distance distribution in galanin in TFE has been obtained here from analysis of the Förster resonance energy transfer rate data without input estimations about the number of expected components nor about their distribution function. The number of degrees of freedom is not fixed, but is automatically determined during the analysis by the constraints and by the regularizer, which adapts itself to the noise level of the data (Provencher 1982). The CONTIN routine used for the analysis automatically selects the best fitting as the compromise between a stable solution and an adequate model.

The model applied for the analysis of the energy transfer by fluorescence decay is restricted (Eq. (2)). The fluorescence decay spectrum in the presence of energy transfer is fitted with a linear combination of the original fluo-

rescence decay spectrum modified by the additional transfer rate giving the function $P(r)$. The function $P(r)$ is represented by a contribution of discrete values of distances resulted from the scanned grid of distances created during the CONTIN analysis. Additionally the amount of the contamination term visible as a distinct decay component not existing in the wild type molecule is included in the analysis as the parameter γ.

The full-width half maxima of the peaks of the recovered distance distribution are 2 Å for R at 16 Å, about 2.5 Å for R at 21 Å and 3 Å for R at 34 Å. The uncertainties of evaluated Förster distances, R_0, as a function of possible extreme orientation factors estimated for the Trp2-DNS-Lys25 system in galanin is in the same range. Most systems studied so far by time-resolved energy transfer exhibit an apparent distance distribution with the substantial width (Cheung et al. 1991; Haas et al. 1975; Haas et al. 1988; Lakowicz et al. 1988), although the average distances from energy transfer measurements are very close to those known from X-ray crystallographic studies. Only some oligopeptides (Haas et al. 1975, Lakowicz et al. 1990) and glycopeptides (Rice et al. 1991; Wu et al. 1991) show narrow distribution widths. In the analysis of those results, however, a given model distribution function around a single distance has been assumed and its parameters fitted. The results obtained in the present study indicate the existence of specific maxima in the distance distribution. In our work we have used the regularisation scheme implemented in the CONTIN program to promote a broad distribution function, and in that way to assure more reliable results if sharp features are present in the final fit only as a true consequence of experimental data. Owing to the restraints intoduced by CONTIN only the directions in parameter space that are required to describe data are free, and the width of recovered maxima should be considered as less reliable in contrast to the maxima positions.

The distance distribution results obtained in this work point to the existence of galanin in TFE in different conformational states with some degree of the dynamic averaging within each class of structures. Galanin in TFE has a highly α-helical structure, but the glycine–proline sequence in the middle of the molecule breaks this into two parts joined by a flexible structural element. The distance between Trp2 and Lys25 is a function of the angle of kink between the two helical fragments and ranges from 35 to 16 Å. The longest Trp2-Lys25 distance found for galanin in TFE would correspond to the slightly kinked conformation characterised by an angle of about 160° between α-helices in the elongated structure. The short distance should correspond to a folded-back structure.

Steric hindrance in these structures may form energetic barriers and additional stabilizing interactions may occur when kinked structures fold, allowing the direct interactions of side chains from distant molecular regions. These appear to be enough to separate extended and kinked structures. The stabilisation should be sensitive to changes in physico-chemical properties of the end of the peptide, and influenced by chromophore labelling. Although separable on the nanosecond time-scale, these structures are in conformational equilibrium on the much slower NMR time-scale, as confirmed by the analysis of energy minimized averaged structures derived from NMR spectroscopy (Wennerberg 1994).

Computer simulations of the molecular dynamics of galanin in water and TFE carried out in order to extend the knowledge of the influence of different solvents on the conformation and stability of the structure (De Loof et al. 1992) have shown that in aqueous solution the molecule initially folded starts to unfold at once but in TFE remains stable on the sub-nanosecond time-scale. The conformation of rat galanin was also studied using Monte Carlo method with energy minimisation (Liwo et al. 1994). The lowest-energy conformation obtained in aqueous solution calculations had a nonhelical N-terminal part packed against the residual helix that extended from Pro 13 toward the C-terminus. In contrast, all of the low-energy conformations in the absence of water were helical, differing only by the extent to which the helix was kinked around Pro13.

It is interesting to note that the conformational equilibrium as reflected by the end-to-end distance distribution differs between DNS- and DNP-labelled peptide. The proportion of the molecules in folded conformations is 79% for the Trp-DNS donor-acceptor pair whereas for Trp-DNP donor-acceptor pair the folded conformations contribute only 13%. This result may correlate with the different affinity to the galanin receptor between these two labelled forms of galanin. It is known that the N-terminal part, residues 1 to 16, is involved in the binding to the receptor and also that the Trp2 is essential for this binding, while the C-terminal part seems not to be directly involved in this interaction, so a direct influence of labelling of this part of the peptide on the binding properties would not be expected. However, galanin derivatised with DNS group may have general physical-chemical properties affected by this relatively large group carrying its dimethylamino group positive charge replacing Lys epsilon-amino group, in contrast to the small, neutral dinitrophenyl residue, as demonstrated in different conformational equilibrium and binding to the receptor.

Conclusions

In this report we have demonstrated the utility of time-resolve fluorescence energy transfer measurements for studying the solution conformation of peptides.

The method applied for the analysis of energy transfer measurements presented in this paper enables us to determine the distance distribution between two fluorophores within a macromolecule with reasonable precision giving the possibility to distinguish different conformations of the biomolecule. Its application at various temperatures, allowing the observation of altered equilibrium states, should prove particularly important. The energy transfer measurements give direct information about long distance separa-

tion of defined residues of the molecule complementary to those attained by NOE, which is limited to short distances and hence may provide supplementary distance constrains for structure determination.

Binding studies carried out on the labelled peptide have indicated that the binding affinity of DNP-galanin is in the same range as for the native peptide. This makes it suitable for distance measurements when binding to its receptor. Folded conformations dominate for DNS-galanin and the binding affinity is significantly lower than of native galanin, while only a minor population of folded conformations of DNP-galanin is formed. The different conformational equilibria may be due to a charge effect related to the DNS-group replacing the Lys epsilon-amino group. In this respect the small, neutral and non fluorescent dinitrophenyl residue seems to be the acceptor of choice for energy transfer measurements on relatively small dynamic structures.

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