The neuropeptide Y monomer in solution is not folded in the pancreatic-polypeptide fold

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Abstract

Fluorescence-labelled analogs of NPY, a 36-amino acid peptide amide, were synthesized by solid-phase peptide synthesis and used for fluorescence-resonance energy transfer studies to investigate the conformation. Energy-transfer efficiency measurements in different media at the concentration of 10 μ M are in agreement with a model of the NPY structure proposed by NMR studies (performed at millimolar concentration) in which the C-terminal part of the molecule adopts an α -helical conformation while the N-terminal part is flexible. According to this model, the α -helix is stabilized by intermolecular hydrophobic interactions because of the formation of dimers. The decrease of the peptide concentration causes a shift of the dimerization equilibrium toward the monomeric form. Energy-transfer efficiency measurements performed at lower concentrations do not support the hypothesis of the folding back of the N-terminal tail onto the C-terminal α -helix to yield the so-called "PP-fold" conformation. This structure is observed in the crystal structure of avian pancreatic polypeptide, a member of the NPY peptide hormone family, and it has been considered to be the bioactive one. Our results complete the structural characterization of NPY in solution at concentration ranges in which NMR experiments are not feasible. Furthermore, these results open the way to study the conformation of the receptor-bound ligand.

Keywords: Conformational changes; dissociation equilibrium; FRET; GPCR; neuropeptide Y; peptide structure in solution; PP-fold

One of the crucial points in the development of new drugs is the understanding of the conformational changes that are performed by a bioactive ligand during the process of the binding to its receptors. Innovative experimental ap-

proaches are required that could shed light into the dynamic process in which a small ligand molecule binds to membrane protein receptors consisting of several hundreds of amino acid residues. A ligand, which has been made the object of many pharmacological and structural studies in the last years, is NPY. This 36-amino acid peptide amide was isolated from porcine brain in 1982 (Tatemoto et al. 1982) and is the most abundant neuropeptide in the central nervous system of mammals (Gray and Morley 1986). The numerous important physiological effects attributed to NPY include the hypothalamic regulation of food intake (Turton et al. 1997; Söll and Beck-Sickinger 2001), of blood pressure (Grundemar and Hakanson 1993), and of the secretion of other hormones (Turton et al. 1997). Several reports have appeared in the last few years that have shown that NPY is involved in epilepsy (Vezzani et al. 1999). All of these physiological effects are mediated by at least six GPCRs named Y_1 , Y_2 , Y_3 , Y_4 , Y_5 , and y_6 (Grundemar 1997; Michel et al. 1998; Cabrele and Beck-Sickinger 2000). In addition

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Abbreviations: Boc, tert-butoxycarbonyl; CD, circular dichroism; dansyl (Dns), 5-dimethylaminonaphthalene-1-sulfonyl; DIC, N,N-diisopropylcarbodiimide; Dpr, α,β -diaminopropionic acid; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G-protein-coupled receptor; HSQC, hetero-nuclear single quantum correlated spectroscopy; HOBt, 1-hydroxy-benzotriazole; HPLC, high-performance liquid chromatography; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene); FRET, fluorescence resonance energy transfer; MALDI-MS, matrix assisted laser desorption ionisation mass spectrometry; NMR, nuclear magnetic resonance; NPY, neuropeptide Y; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PP, pancreatic polypeptide; PYY, peptide YY; tBu, tert-butyl; TFA, trifluoroacetic acid, TFE, 2,2,2-trifluoroethanol; Trt, trityl; UV, ultraviolet.

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to NPY, PYY and PP are the two naturally occurring peptide ligands for the Y-receptor family (Fig. 1).

The resolution of the crystal structure of avian pancreatic polypeptide (Blundell et al. 1981) provided a three-dimensional model, which, on the basis of the high homology between the primary structures, was also applied to NPY (Allen et al. 1987). In this model, a specific structure was identified, the so-called PP fold (Fig. 1). The N-terminal part of the molecule (residues 1–8) adopts a polyproline type II helical conformation, and residues 9 to 13 form a loop that allows the polyproline helix to fold back onto an α -helix encompassing residues 14 to 31. This leads to a stabilization of the two different helices by hydrophobic interactions. The last five amino acids in the C-terminal tail are flexibly arranged. The hypothesis of the PP fold for NPY was confirmed by NMR data (Darbon et al. 1992), and other reports (Cowley et al. 1992; Mierke et al. 1992; Monks et al. 1996), also based on NMR data, highlighted the existence of dimers, in which two α -helices that belong to two different molecules are facing and stabilizing each other in solution. In this model, the N-terminal part of the molecule is flexible. Recently, by [^{15}N , ^1H]-HSQC NMR experiments, it was possible to characterize the structure of micelle-bound NPY and to compare it with that of unbound NPY (Bader et al. 2001). This work has definitely confirmed the existence of dimers and the absence of the N-terminal polyproline helix in the examined millimolar concentration range. Furthermore, it has been shown that the same hydrophobic face of the α -helix is used for both the dimerization interface and to bind to the membrane surfaces. The use of a spin-labelled NPY analog has allowed us to prove the concomitant existence of a parallel and an antiparallel orientation of the two α -helical segments in the

dimer formation. The only significant structural difference between micelle-bound and free NPY is that the C-terminal residues 32 to 36, which are flexible in solution, become α -helical in the membrane-bound molecule. Although NMR is nowadays the technique that provides the richest set of information to investigate the structure of a peptide in solution, there are some limitations that reduce its effectiveness in biological systems. To obtain a sufficient intensity of the NOE signals, it is necessary to use NPY samples in the millimolar range. Sedimentation equilibrium (Minakata et al. 1989), circular dichroism (Nordmann et al. 1999), and fluorescence studies (Cowley et al. 1992) provided an estimation of the dissociation constant (K_D) for the NPY dimer of $\sim 2 \mu\text{M}$. Accordingly, NPY is completely dimerized at the millimolar concentrations used in NMR experiments. NPY *in vivo* is active at nanomolar concentrations, where it is a monomer (Minakata et al. 1989). Therefore, the characterization of NPY structure in solution at low concentrations, where the monomer content becomes predominant, necessitates other approaches and techniques besides NMR. Because biological data indicate a close contact between the N and C termini at the receptor (Rist et al. 1996; Cabrele and Beck-Sickinger 2000), the hypothesis has been made that NPY adopts the PP-folded conformation by becoming monomeric (Nordmann et al. 1999). The dimerization at high concentration was postulated to be a way to store the peptide in an inactive form and to allow a slow release of the active monomer.

In this study, we investigated the conformation of NPY in solution at a concentration at which the monomeric form becomes predominant. Our main focus was to ascertain whether the N-terminal tail, which is flexible in the dimer, folds back onto the C-terminal α -helix to give the characteristic PP fold. Among the different experimental approaches, we found that the use of FRET (Stryer 1978) is the most suitable technique. Electronic excitation energy can be transferred from a donor to an acceptor in a dipole-dipole energy transfer process. The energy transfer efficiency can be quantified by time-resolved or steady-state methods (Wu and Brand 1994) and, according to Förster's theory, it can be used to estimate the distance between donor and acceptor (Stryer 1978). We have synthesized a set of fluorescence-labelled NPY analogs in which the fluorescence donor is provided by the indole group of Trp and the acceptor by the dansyl moiety covalently linked to the β -amino group of a Dpr residue. Trp was always located in proximity to the C-terminal part of the molecule, whereas the position of dansyl was varied. Energy-transfer efficiency measurements performed on the different analogs allowed us to monitor the peptide folding in different buffer solutions and at different concentrations, and to complete the characterization of the NPY structure in a low concentration range, where NMR data are not available.

Fig. 1. Three-dimensional structure of the characteristic PP fold derived from the crystal structure of avian pancreatic polypeptide (Blundell et al. 1981) and the primary structures of human NPY, PYY, and PP.

Results

Peptide design and synthesis

The fluorescence analogs investigated in these studies are based on the sequence of porcine NPY (pNPY), which differs from the human sequence by a Leu residue in position 17 instead of a Met. Because of the five Tyr residues located in positions 1, 20, 21, 27, and 36, native pNPY is an intrinsically fluorescence-active molecule. The intrinsic fluorescence cannot be used for FRET studies because the signal of the different Tyr residues cannot be distinguished. On the other hand, the introduction of “artificial” fluorescence probes has to match the necessity of preserving the native peptide structure and their position has to be as close as possible to the peptide scaffold to reduce flexibility. The choice of using indole and dansyl as a donor/acceptor pair is based on the relatively small size of the fluorophore groups and on the fact that they can be placed at a short distance from the peptide backbone. The indole group is naturally attached to the Trp C^β-atom, whereas the dansyl group can be attached to the side-chain β-amino group of the amino acid Dpr (Fig. 2). To allow a rational interpretation of the energy transfer data, we kept the position of one of the two members of the FRET pair (Trp) fixed while we varied that of the other along the sequence (Fig. 2). Trp was always introduced in the C-terminal part of the α-helix in position 31 (but once in position 29), whereas Dns(Dpr) was located at position 2, corresponding to the beginning of the proline helix of the putative PP fold, or at the beginning (position 12) or in the middle (position 24) of the α-helical part (Bader et al. 2001). Two mono-labelled analogs [Dpr(Dns)]²-NPY and [Trp³¹]-NPY were used as reference compounds in the fluorescence studies.

The synthesis of the labelled analogs was entirely performed by the solid-phase approach. ivDde was used to selectively protect the β-amino-group of Dpr (Chhabra et al. 1998). The assembly of the full-length, fully protected peptides was performed on a Rink-amide resin using the Fmoc strategy. The N-terminal Tyr was introduced by Boc protection because of the instability of the Fmoc group under the cleavage conditions of ivDde. After the selective re-

moval of ivDde with hydrazine, dansylation was performed with Dns-Cl, followed by cleavage of the peptide and subsequent purification and analysis (Table 1).

Circular dichroism

The CD spectra of the fluorescence-labelled analogs and of the native pNPY were recorded in water at 10 μM peptide concentration (Fig. 3A). They all show an identical profile characterized by a negative maximum at 209 nm and a pronounced shoulder at 220 nm. Slight differences in the intensity of the signal are observed. To investigate the effect of the formation of heterodimers on the peptide structure, we recorded a CD spectrum of a solution containing 5 μM pNPY and 5 μM [Dns¹²-Trp³¹]-pNPY (Fig. 3B). This mixture showed the same profile as pure NPY.

Binding properties

Binding assays on three different cell lines, each selectively expressing either the Y₁, the Y₂, or the Y₅ receptors, were performed by displacement of ³H-propionyl-NPY, as reported in Materials and Methods. The K_i values reported in Table 1 show that there is a partial retention of the binding properties of native pNPY. The concomitant presence of Trp in the C-terminal part of the α-helix (positions 29 or 31) and the dansyl group in position 12 or 24 generally determines an increase of the K_i values for all of the receptor subtypes.

FRET measurements

Fluorescence measurements were performed in different solvents, including 10 mM acetate buffer (pH 3), 0.1 M phosphate buffer (pH 7), 10 mM carbonate buffer (pH 8), 4 M guanidinium chloride in water, and 50% TFE in water. The concentration range examined in the fluorescence studies went from 10 μM to 2.5 μM; the low solubility of NPY at pH 7 and pH 8 impaired measurements at higher concentrations, whereas the instrumental sensitivity represented a limit for the experiments at higher dilution. Because the

Fig. 2. Sequences of the fluorescence-labelled pNPY analogs used in the FRET studies. X represents the N-β-dansyl Dpr. The second fluorophore (Trp) is marked in bold as W.

Table 1. Analytical and binding data of the fluorescence labeled NPY analogs^a

Peptide	M.W. (a.m.u.)		K _i (nM)		
	[M+H] ⁺ Theoretical	[M+H] ⁺ Experimental	Y ₁ SK-N-MC	Y ₂ SMS-KAN	Y ₅ BHK
pNPY	4252	4251	0.03	0.12	0.56
[Dpr(Dns) ²]-pNPY	4474	4477	3.2 ± 2.9	0.198 ± 0.054	58 ± 14
[Trp ³¹]-pNPY	4326	4326	29 ± 4	5.9 ± 4.9	55 ± 14
[Dpr(Dns) ² ,Trp ³¹]-pNPY	4547	4549	>1000	14.5 ± 6.65	25.4 ± 20.9
[Dpr(Dns) ¹² ,Trp ³¹]-pNPY	4573	4576	>1000	176 ± 49	84.5 ± 65.8
[Dpr(Dns) ²⁴ ,Trp ²⁹]-pNPY	4530	4530	49 ± 29	105 ± 52	>1000

^a Molecular weights were measured by MALDI-MS. Binding assays on three different cell lines (SK-N-MC, SMS-KAN, BHK expressing respectively the hY₁, hY₂, and rY₅ receptors) were performed by competition assays as reported in Materials and Methods. Each experiment was repeated three times. IC₅₀ values were determined performing a Scatchard plot. K_i values were calculated from the corresponding IC₅₀ values (Y₁ receptor, K_D = 0.18 nM; Y₂ receptor, K_D = 0.4 nM; Y₅ receptor, K_D = 2.4 nM). The K_i values of NPY were obtained from the IC₅₀ data listed in the literature (Cabrele and Beck-Sicking 2000).

dissociation constant of NPY was determined to be ~2 μM (Minakata et al. 1989), the monomer content increases from 27% at 10 μM to 46% at 2.5 μM. The measured concentration range was therefore sufficient to evaluate the conformational changes induced by the transition from dimer to monomer. The error because of concentration differences was estimated to be 10%. Because the distance between the chromophores (R) depends on the sixth power of the energy transfer, the error in R was estimated to be 6%. It has been shown (Schiller 1972) that the interference of Tyr residues in the FRET process between Trp and dansyl can be eliminated by irradiating at 293 nm because the Tyr absorption is negligible at this wavelength. The fluorescence emission spectra obtained by excitation at 293 nm (Fig. 4) present a first peak at 350 nm corresponding to the indole emission and a second one, whose maximum varies between 480 and 525 nm, according to the buffer used and the peptide con-

centration (data not shown), produced by the emission of the dansyl group. The spectrum of the peptide [Dns²-Trp³¹]-pNPY shows that the energy transfer results in a quenching of the emission signal of the donor (Trp) and an enhanced emission of the acceptor (dansyl). It is known that the intensity of the emission of a fluorophore depends also on the polarity of the environment (Turcatti et al. 1997). The residues located at the dimerization interface of the α-helical part of the NPY molecule are expected to contact a more hydrophobic environment than those located in the flexible N-terminal tail. Because the position of the dansyl acceptor is varied in this set of analogs, to evaluate the energy transfer efficiency, it is more convenient to monitor the quenching of the indole donor whose position is always kept constant in the C-terminal part of the α-helix. Energy transfer efficiency is also influenced by the reciprocal orientation and mobility of the donor and acceptor chromophores (Stryer 1978). Because fluorescence anisotropy values pro-

Fig. 3. (A) CD spectra of NPY and four of the fluorescence-labelled peptides in water at the concentration 10 μM. (B) Comparison of the CD spectra of pNPY (10 μM in water) and a mixture of 5 μM pNPY and 5 μM [Dns¹²,Trp³¹]-pNPY measured under the same conditions.

Fig. 4. Fluorescence emission spectra of [Dns²]-pNPY, [Trp³¹]-pNPY, and [Dns²,Trp³¹]-pNPY at the concentration 10 μM and of a mixture of [Dns²]-pNPY 10 μM and [Trp³¹]-pNPY 10 μM. All spectra were recorded in water with an excitation wavelength of 293 nm.

duced by dansyl chromophore located in positions 2, 12, and 24 (data not shown) are all largely below the critical value of 0.2 (Clegg 1992), indicating that at least one of the two members (dansyl) of the FRET pair is sufficiently free to rotate, we conclude that the orientation factor does not play an important role in the observed variation of the energy transfer efficiencies. This finding is not surprising considering that there are two single bonds connecting the dansyl group to the Dpr C α atom that allow free rotation. Further support for the validity of the statement concerning the independence of the FRET effect from the orientation factor is given by the fact that most of the recent FRET studies on biological systems in solution (Schneider and Schepartz 2001; Toth et al. 2001) do not even consider the effect of the orientation factor on the values of the energy transfer efficiencies. The energy transfer efficiency (E) can be derived from the following equation

$$E = 1 - F_{DA}/F_D \quad (1)$$

where F_{DA} represents the fluorescence of the donor in presence of the acceptor, and F_D is the fluorescence of the donor itself. In this study, F_{DA} and F_D were obtained from the intensity of the indole emission whose maximum is located around 350 nm; F_D was derived from the spectrum of the analog [Trp³¹]-pNPY, and F_{DA} resulted from the emission of the different double-labelled peptides. To minimize the experimental errors, we used integrated spectral regions extending from 345 to 355 nm. It is possible to calculate the distance R between the two fluorophores using the equation

$$R = R_0[(1 - E)/E]^{1/6} \quad (2)$$

where R_0 is the so-called Förster's radius, which depends on the FRET pair and on the solvent.

Information on the tertiary structure of NPY is complicated by the presence of dimers in which the two NPY molecules can have a parallel and an antiparallel orientation (Bader et al. 2001). Energy transfer between donor and acceptor can result from inter- and intramolecular interactions. Accordingly, we measured the fluorescence emission spectrum of an aqueous solution containing 10 μ M [Dns²]-pNPY and 10 μ M [Trp³¹]-pNPY (Fig. 4). These two singly-labelled analogs may form heterodimers in solution that will show only intermolecular quenching. However, in the mixture of 10 μ M [Dns²]-pNPY and 10 μ M [Trp³¹]-pNPY, the intensity of the signal corresponded exactly to the sum of the spectra of the two peptides taken alone at a concentration of 10 μ M each. A 10- μ M solution of the peptide [Dns², Trp³¹]-pNPY, however, showed a marked quenching of the Trp emission and a corresponding increase of the dansyl signal, as expected in FRET (Fig. 4). These results clearly

show that the dansyl group in position 2 and Trp in position 31 can only interact by intramolecular energy transfer.

To test whether FRET is a useful technique to monitor structural changes in NPY, we performed FRET experiments by using 50% TFE as the solvent mixture. The structure of NPY in 50% TFE has already been investigated by NMR (Mierke et al. 1992). Energy transfer efficiencies were measured for the three double-labelled analogs at three different concentrations (10 μ M, 5 μ M, and 2.5 μ M) (Fig. 5A). The observed energy transfer rank order for all concentrations is [Dns²,Trp³¹]-pNPY < [Dns¹²,Trp³¹]-pNPY < [Dns²⁴,Trp²⁹]-pNPY. Furthermore, the energy transfer efficiencies do not significantly change by varying the concentration. We then compared the energy transfer efficiencies (E) of the three different analogs in different buffers (4 M guanidinium chloride, 50% TFE in water, acetate buffer at pH 3, carbonate at pH 8, and phosphate buffer at pH 7) at the concentration of 10 μ M (Fig. 6). Considering

Fig. 5. Measured energy transfer efficiencies for the peptides [Dns²,Trp³¹]-pNPY, [Dns¹²,Trp³¹]-pNPY, and [Dns²⁴,Trp²⁹]-pNPY at the concentrations 10 μ M, 5 μ M, and 2.5 μ M in TFE/water 1:1 (A), in acetate buffer at pH 3 (B) and in phosphate buffer at pH 8 (C).

Fig. 6. Energy transfer efficiencies (E) for the peptides [Dns²,Trp³¹]-pNPY, [Dns¹²,Trp³¹]-pNPY, and [Dns²⁴,Trp²⁹]-pNPY at the concentration 10 μ M in 4 M guanidinium chloride, TFE/water 1:1, acetate buffer at pH 3, carbonate buffer at pH 8, and phosphate buffer at pH 7. The corresponding theoretical E values, calculated for the PP-fold structure in solution that use Förster's radius for the Trp-dansyl pair in phosphate buffer at pH 7 (24.5 Å), produce a rank order that significantly differs from the experimental ones. This is expected from the dissociation constant of the NPY dimer in solution, which excludes the predominance of PP-folded NPY monomer at these concentrations.

a dissociation constant of 2 μ M (Cowley et al. 1992; Minakata et al. 1989), the NPY dimer should dominate. Accordingly, these FRET values are compatible with the NMR structure of the NPY dimer (Bader et al. 2001). The data are presented in terms of energy transfer efficiencies because only R_0 at pH 7 in 0.1 M phosphate buffer is known (24.5 Å). The lowest values for the FRET efficiencies are observed in guanidinium chloride, as expected because the secondary structure should be essentially disrupted. The observed rank order $E(2-31) \cong E(12-31) \ll E(24-29)$ is in agreement with an unordered peptide and the observed $E(24-29)$ derives from the vicinity of the probes in the primary sequence. In 50% TFE and at pH 3 the rank order is $E(2-31) < E(12-31) < E(24-29)$. This kind of ranking seems to be typical also of the measurements at pH 7 and pH 8, where, however, we observed a much stronger quenching of the Trp signal for all three analogs. This finding makes the evaluation of the energy transfer efficiencies difficult because of the larger experimental errors that result from the low intensity of the fluorescence signal.

To monitor the effect on the peptide structure of a shift of the NPY dissociation equilibrium toward the monomer form, we performed FRET measurements at lower concentrations as well. Figure 5, B and C, shows that, at pH 3 and pH 8, a change in concentration from 10 μ M to 5 μ M and 2.5 μ M causes a drop in the E values that is most significant for the analog [Dns²,Trp³¹]-pNPY. Table 2 shows the Trp-Dns distances calculated on the basis of energy transfer

efficiencies for the analog [Dns²,Trp³¹]-pNPY in 0.1 M phosphate buffer at pH 7. Under these conditions, the value of Förster's radius R_0 is known to be 24.5 Å (Schiller 1972). The resulting distances have to be considered as averaged over the monomer and dimer populations. Furthermore, the final value of R is averaged over the large set of possible distances between the two fluorophores that are allowed by the flexibility of the N-terminal part of the peptide chain.

Discussion

Conservation of the native NPY conformation and binding properties

To investigate the structure of NPY in solution by FRET, we replaced two residues. The exchange of some of the residues of the native NPY sequence with others that do not necessarily share the same conformational preferences might result in a destabilization of the native peptide conformation. To study the structure conservation, we performed a CD study. The identical profile of the CD spectra suggests that the native NPY structure in the dimeric form is not altered in the fluorescence-labelled analogs. Rather than by the modification of the native peptide structure, the loss of affinity observed for the fluorescence-labelled analogs (Table 1) may therefore be explained by the hydrophobicity and relative bulkiness of the indole and dansyl moieties, which might disturb the interaction with the receptor binding site.

Monomer-dimer

The analyses of the structure of monomeric NPY in solution and consequently the structure that interacts with the receptor is still an unsolved problem. To circumvent high concentrations required for NMR studies, we performed FRET experiments using double-labelled NPY analogs. Although the sensitivity did not allow measurements when solely the monomer was present (<5 nM), we could successfully work

Table 2. Distances between Dns² and Trp³¹ according to FRET measurements^a

Concentration (μ M)	Distance (Å)	Monomer %
10	20 $\pm$ 1	27
7.5	22 $\pm$ 1	30
5	23 $\pm$ 1	36
2.5	27 $\pm$ 2	46

^a Solutions of the peptides [Dns², Trp³¹]-pNPY and [Trp³¹]-pNPY in 0.1 M phosphate buffer (pH 7) at different concentrations were excited at 293 nm. The intensities of the Trp emission signal of the two peptides at each concentration value were used to calculate the distances according to (Stryer 1978). The percentage of monomer was calculated by assuming a value of 2 μ M for the dimer dissociation constant K_D .

in the range of 10 μ M (27% monomer) and 2.5 μ M (46% monomer). The differences between these structures should clearly point to the direction of the structural changes that occur when the dimer dissociates. The forces that drive the equilibrium in solution between monomer and dimer are the hydrophobic interactions that are necessary to stabilize an α -helix in water (Zimm and Bragg 1959). The importance of this kind of interaction has been recently underlined by the development of relatively short peptides (23 residues) based on the zinc-finger motif, which adopts a defined tertiary structure even in the absence of metal ions (Struthers et al. 1996). These $\beta\beta\alpha$ peptides consist of a β -hairpin and an α -helical region, respectively located in the N- and in the C-terminal parts of the sequence, connected by a flexible loop that allows the β -hairpin to fold back onto the helix and to stabilize it by hydrophobic interactions. The removal of some residues from the loop region hampers the folding back of the peptide, thus forcing it to oligomerize to stabilize the α -helix (Mezo et al. 2001).

Evidence for intramolecular energy transfer

The absence of intermolecular energy transfer between Dns² and Trp³¹, as shown in Figure 4, can be explained by considering the spatial disposition of the two NPY molecules in the dimer. If the dansyl group is located in position 2 at the end of the flexible N-terminal tail, two intermolecular Trp–Dns distances will be possible according to the two possible orientations of the dimers (Fig. 7). The shorter distance takes place when the two molecules have an anti-parallel alignment; the absence of intermolecular quenching can be due to the fact that, even in the case of the shorter Trp–Dns distance, the space between the axes of the two α -helices is too large to allow this kind of interaction. Considering that the aim of this study is to investigate the hypothesis of the existence of the PP fold at low concentrations, which is characterized by the folding back of the N-terminal part of the molecule onto the C-terminal one, it is not relevant to determine whether the energy transfer

Fig. 7. The two possible orientations of the NPY dimers and the positions of the fluorescence probe according to Bader et al. (2001).

efficiencies between position 12 and 31 and between positions 24 and 29 are entirely due to intramolecular interaction or not, because an eventual folding back of the N-terminal tail to give the PP fold would not dramatically influence these two distances.

FRET studies in 50% TFE

We decided to perform FRET experiments in 50% TFE because CD experiments showed a stabilizing effect of TFE toward the α -helix of NPY, which reaches its maximum with 50% trifluoroethanol, whereas no further increase in helicity is observed between 50% and 90% TFE (Mierke et al. 1992). A remarkable aspect of the FRET studies performed in 50% TFE is that for all three analogs the energy transfer does not significantly change on dilution of the peptide solution. This finding reflects the independence toward concentration changes observed in the CD signal in water–TFE mixtures (Mierke et al. 1992) and proves that the structure of NPY is not affected by dilution. Accordingly, absence of aggregation has been assumed. The observed order for the energy transfer efficiencies (E) measured in 50% TFE (Fig. 5A) is $E(31-2) < E(31-12) < E(29-24)$ and agrees with the solution structure of human NPY, which has been determined in a mixture of 90% TFE and 10% D₂O by NMR experiments (Mierke et al. 1992). The results of the NMR study show that in this solvent the α -helix is extended from Arg¹⁹ to Glu³⁴, whereas no regular structure was observed in the N-terminal part of the molecule. As the C-terminal half of the peptide adopts an α -helical conformation, the distance between residues 24 and 29 must be shorter than that between residues 12 and 31, and, consequently, in the first case the energy transfer has to be more efficient. According to the NMR model proposed by Mierke and colleagues, both residues 2 and 12 are located within the N-terminal flexible part and thereby possess wide motional freedom with respect to the rigid α -helix; in the cases of [Dns²,Trp³¹]-pNPY and [Dns¹²,Trp³¹]-pNPY, the measured energy transfer efficiencies result from an ensemble of very different Trp–Dns distances. The length of the flexible tether, which connects residue 12 to the α -helix, is smaller than in the case of residue 2, resulting in a shorter average spatial distance for the 12–31 pair than for the 2–31 pair. This is reflected by a higher energy transfer efficiency for [Dns¹²,Trp³¹]-pNPY than for [Dns²,Trp³¹]-pNPY. Because of the analogies shown in CD measurements and the agreement with the NMR studies, we can conclude that FRET studies are suitable to monitor the changes in the structure of NPY.

FRET studies in guanidinium chloride

In 4 M guanidinium chloride, NPY is completely denatured (CD data not shown). The observed order for the en-

ergy transfer efficiencies (E) measured under these conditions is $E(2-31) \cong E(12-31) \ll E(24-29)$ (Fig. 6). The total absence of the α -helix, even in the C-terminal part, allows residues 2 and 12 to reach distances from the C terminus that cannot be reached when the helical frame is present. This results in much lower values for $E(2-31)$ and $E(12-31)$ than for $E(24-29)$. Assuming that each of the single bonds that forms the peptide chain has an average length of 1.5 Å (Jakubke 1996a), the largest reciprocal distance that can be achieved by residues 24 and 29 when the peptide chain has a completely extended conformation between these two residues would be similar to the R_0 value reported in the literature for the dansyl-Trp pair in guanidinium chloride 6 M (23.6 Å) (Schiller 1972). According to equation (2), R_0 can be defined as the distance between donor and acceptor that determines an energy transfer efficiency of 0.5; it is therefore reasonable that the experimental value of E obtained for the peptide [Dns²⁴-Trp²⁹]-pNPY in 4 M guanidinium chloride does not dramatically differ from 0.5. From Figure 6 we can see that this value is about 0.6, indicating an average distance between Dns²⁴ and Trp²⁹ that is shorter than the maximal distance allowed by the peptide chain.

FRET in different buffers

The energy transfer efficiencies relative to the three doubly labelled analogs in the same medium represent the energy transfer efficiency order typical of that medium. The differences in the energy transfer efficiency orders observed in different buffers (Fig. 6) have to be explained by different structures of the peptide in the different media rather than by the different value of Förster's radius (R_0), which may account only for the difference in the relative FRET efficiency intensity of the same analog in different media. In fact, although R_0 for the Trp-dansyl pair is buffer dependent only from 20 to 26 Å (Schiller 1972), (as already mentioned the energy transfer efficiency depends on the sixth power of R_0), small changes in R_0 produce large variations in the experimental E values. As discussed for the case of TFE 50% (*vide supra*), the rank order $E(31-2) < E(31-12) < E(29-24)$ observed at pH 3 is in accordance with the model proposed by NMR studies (Bader et al. 2001) in which the N-terminal part is flexible and the C-terminal segment is α -helical. The fact that the difference between the values of $E(31-2)$, $E(31-12)$, and $E(29-24)$ seems to be more pronounced at pH 3 than at pH 7 and pH 8 (Fig. 6) can be explained by a slight prolongation of the α -helical tract toward the N terminus at more basic pH values. Remembering the geometrical features of standard α -helix (Jakubke 1996b), a hypothetical prolongation of the helical tract in this direction would cause an increase in the length of the α -helical axis of 1.5 Å but would reduce the maximal length of the flexible N terminal tether by about 4.5 Å, which

roughly corresponds to the length of the three single bonds for each amino acid in a fully extended conformation. The increase in the helical length toward the N terminus at more basic pH should therefore correspond to a decrease in the effective distance between residues 2 and 31. In accordance with this hypothesis, the mean residue molar ellipticity ($[\theta]_R$) at 222 nm for a 10- μ M NPY solution is lower at pH 8 ($-7319 \text{ deg cm}^2 \text{ dmol}^{-1}$) than at pH 3 ($-6688 \text{ deg cm}^2 \text{ dmol}^{-1}$) (CD spectra not shown) that indicate a higher α -helical content at pH 8. The hypothesis that the larger $E(31-2)$ at pH 7 and pH 8 than at pH 3 might be explained by a higher content of dimer and a consequent higher rate of intermolecular energy transfer has to be excluded because we have shown that the FRET effect between these two positions is only of intramolecular origin. This observation further supports our view that FRET is an appropriate method to investigate NPY structure. As already stated, NPY exists at 10 μ M mainly in the dimer form. Therefore we do not expect that the signal produced at this concentration would correspond to the PP-folded monomer, which should prevail at lower concentrations. However, to answer the question on what we will expect the E pattern to be if NPY is folded in the PP structure, we had used the available data from the crystal structure of avian pancreatic polypeptide (aPP) to simulate the corresponding E values. These values were calculated by using the corresponding C^α - C^α interresidual distances obtained from the crystal structure of the avian pancreatic polypeptide (Blundell et al. 1981) R_0 was assumed to be 24.5 Å, as calculated for the Trp-dansyl FRET pair in 0.1 M phosphate buffer at pH 7 (Schiller 1972). We further collected data from fluorescence anisotropy of a 10- μ M solution of the peptide [Dns²⁴,Trp²⁹]-pNPY (data not shown), which indicate that even when inserted in the α -helical segment, where the scaffold rigidity should be similar to that of a hypothetical polyproline helix, the dansyl group conserves a large mobility. We also used the value of R_0 that was calculated for the dansyl-Trp pair in the hypothesis of a "dynamic random orientation" of the chromophores (Schiller 1972) for the case of the PP fold. The PP-folded conformation should produce a pattern in which $E(31-2) \cong E(29-24) > E(31-12)$ (Fig. 6) because of the folding back of the N-terminal part onto the α -helix. As observed in Figure 5, a decrease of the peptide concentration that yields an increase in the percentage of molecules in the monomer form does not seem to shift the typical pattern $E(31-2) < E(31-12) \leq E(29-24)$ toward the pattern expected for a PP-fold structure $E(31-2) \cong E(29-24) > E(31-12)$. Because of the absence of intermolecular quenching between positions 2 and 31, the decrease of $E(31-2)$ cannot be due to the dimer dissociation itself, but to a change in the structure and flexibility of the monomer promoted by the dissociation. The fact that the decrease of $E(29-24)$ and $E(31-12)$ on decrease of the concentration would be less relevant than for $E(31-2)$, especially at pH 8, may be inter-

preted as a clue to the fact that intermolecular quenching does not play a significant role even for the peptides [Dns¹²,Trp³¹]-pNPY and [Dns²⁴,Trp²⁹]-pNPY. This experimental result can be explained by the hypothesis that, as the peptide reaches the monomer form, the C-terminal α -helix partially preserves its stability, especially at slightly basic pH values. The average distance between residues 2 and 31 at pH 7 increases hand in hand with dilution (Table 2). This is not compatible with the hypothesis that the NPY monomer assumes the PP-fold conformation, which would lead the two termini to a closer position. The obtained values for R are all above 20 Å. Even considering that there is a flexible spacer between the C α atoms and the fluorophores, which increases the distance between dye and backbone, these values are far away from the value of 9 Å observed to be the distance between the two C α atoms of residues in position 2 and 31 in the crystal structure of avian pancreatic polypeptide (Blundell et al. 1981).

Conclusions

The results presented in our study suggest that the dissociation of the NPY dimer is not accompanied by a folding back of the N-terminal segment on the C-terminal amphipathic α -helix. CD spectra of human NPY (hNPY) show a loss of ordered structure at lower concentrations (Nordmann et al. 1999) that indicates a smaller helical content in the monomer form. Because this decrease in helicity is accompanied by a more dramatic effect on *E* (31–2) than on *E* (31–12) and *E* (29–24), it is reasonable to conclude that the destabilization affects more the N-terminal part of the α -helix. We interpret such experimental data by proposing that a decrease in concentration does not yield the PP fold as postulated, but rather that monomeric NPY prefers a less ordered structure in which the N-terminal part of the α -helix is more destabilized. We propose that this monomeric, less ordered structure, defined as “dynamic state” (Nordmann et al. 1999), is present in minor percentage at higher concentration in equilibrium with the predominant dimer and seems not to guide receptor selection. This experimental evidence confirms therefore the importance of the binding of NPY to cellular membranes as a fundamental step that induces the required conformation before the binding to the receptors. Our study shows that the determination of the structure of a peptide ligand performed in solution in the absence of receptors is not sufficient to determine the structural features characterizing the binding process. Furthermore, we could show that FRET is a useful and convenient technique to investigate the structure of small proteins in highly diluted concentrations where NMR cannot be applied. This frequently happens for molecules with monomer–dimer equilibrium and dissociation constant in the μ M-range, such as chemokines and cytokines. Because we can investigate the conformation at biologically relevant

concentrations by fluorescence techniques, the exploitation of additional fluorescent dyes, which are active at longer wavelengths, is currently in progress in our laboratory. This will allow us to study the conformation of NPY on the surface of the cellular membranes and bound to the receptors. In any case, we do not exclude that, at least for some of the receptor subtypes, the PP fold might be the structure of the receptor-bound NPY.

Materials and methods

Peptide synthesis

Peptides were synthesized by automated solid-phase peptide synthesis following the Fmoc strategy, as previously described (Rist et al. 1996) using an automatic peptide synthesizer (Syrro II, Multi-synTech). The side chains of Asp, Glu, Thr, and Tyr were protected by *t*Bu, and those of Asn, Gln, and His were protected by Trt. Pmc was chosen as a side-chain protecting group for Arg, and Boc was chosen for Lys. 4-(2',4',-dimethoxyphenyl-Fmoc-amino-methyl)phenoxy resin with polystyrene-1% divinylbenzene as a polymer matrix was used to obtain peptide amides. The resin loading varied between 0.45 and 0.55 mmol/g. Couplings were performed using HOBt/DIC as activating reagents. Each coupling reaction was performed at room temperature in dimethylformamide (DMF) using a 10-fold excess of Fmoc-amino acid and coupling reagents. The coupling reagents concentration was 0.5 M. Each coupling step was performed under moderate shaking of the resin suspension over 30 min and was performed twice using the same conditions. No capping was applied. The N-terminal amino acid to be attached to the peptide chain (Tyr¹) was α -amino protected by Boc group. Fmoc-Dpr(ivDde)-OH (Novabiochem) was inserted in position 2, 12, or 24 according to the desired analog. The ivDde group was removed from the full-length, fully protected, and resin-bound peptides by treatment with 2% hydrazine in *N,N*-dimethylformamide, changing the solvent aliquot every 10 min. The cleavage was monitored following UV absorption at 300 nm. The dansyl group was coupled to the side-chain-free Dpr by suspending the resin in 600 μ L dichloromethane solution containing three equivalents of dansyl chloride (Fluka). After 1 h stirring at room temperature, one equivalent *N,N*-diisopropylethylamine, was added and the solution was kept under the same conditions overnight. The resin was washed several times with dichloromethane and dried under vacuum. A Kaiser test (Kaiser et al. 1970) was performed to check the completion of the reaction. The peptides were cleaved by treating the resin with 1 M trimethylbromosilane in TFA/ethanedithiol/thioanisole (9/7/3 v/v) for 1.5 h. After removal of the resin by filtration, the peptide was precipitated from diethyl ether at 0 °C, washed several times with cold diethyl ether, and dried. The crude product was dissolved in *tert*-butanol/water (1:4 w/w) and lyophilized. The purification (purity >95%) was performed by reverse-phase semipreparative HPLC on a Vydac 218TP510 column (C₁₈, 5 μ M, 10 $\times$ 250 mm, Vydac). The eluting system was 0.08% trifluoroacetic acid in acetonitrile (A) and 0.1% trifluoroacetic acid in water (B), applying a linear gradient from 20 to 70% A over 30 min at the flow rate of 2.5 mL/min. Characterization of the peptides (data reported in Table 1) was performed by MALDI-MS using a Voyager DE RP (PerSeptive Biosystems).

UV absorption and fluorescence measurements

Stock solutions of the different analogs were prepared in pure water in the concentration range 0.1–1 mM. The exact concentra-

tion was determined by UV absorption using an EZ210 UV/Vis spectrophotometer (Perkin Elmer). For the dansyl-containing peptides, the dansyl absorption maximum at 330 nm was monitored using $\epsilon_{330} = 3400 \text{ M}^{-1} \text{ cm}^{-1}$ (Chen 1968) as the extinction coefficient. For the analog [Trp³¹]-pNPY, the absorption at 280 nm was considered; the average extinction coefficient of Tyr and Trp at this wavelength were determined to be 1480 and 5540 $\text{M}^{-1} \text{ cm}^{-1}$, respectively (Mach et al. 1992). Because this analog contains one Trp and five Tyr residues, we used for it $\epsilon_{280} = 12940 \text{ M}^{-1} \text{ cm}^{-1}$. According to the concentration of the stock solutions, the probes for the fluorescence measurements were prepared by dilution of a stock solution aliquot in the appropriate buffer. The following buffers were used: 10 mM acetate buffer at pH 3, 0.1 M phosphate buffer at pH 7, 10 mM carbonate buffer at pH 8, 4 M guanidinium chloride in water, 50% TFE in water. Fluorescence spectra were recorded on a LS50B luminescence spectrometer (Perkin Elmer), exciting at 293 nm and collecting the emission signal between 300 and 550 nm. Measurements were performed at 25°C. Excitation and emission slit widths were varied from 3 to 5 nm according to peptide concentration. Quartz cells with a path length of 10 mm were used. The spectra were corrected for the buffer emission. For the determination of the energy transfer efficiencies according to equation (1), integrated spectral regions between 345 and 355 nm were considered. The numerical integration of the spectra between 345 and 355 nm was performed using the programs Microsoft Excel 2000 and Microcal Origin 3.5.

Circular dichroism

CD spectra were recorded on a J 715 dichrograph (Jasco). The peptide solutions were prepared as described for the fluorescence measurements. Quartz cells with a path of 5 mm were used. Each measurement was repeated three times and the resulting signals were averaged. Response time was set to 2 s with a scan speed of 0.2 nm/min; sensitivity was set to 10 mdeg and step resolution to 0.2 nm. The mean-residue molar ellipticity $[\theta]_R$ was expressed in $\text{deg cm}^2 \text{ dmol}^{-1}$.

Cell culture

SK-N-MC cells (neuroblastoma, hY₁) were cultivated in minimum essential medium (MEM) with Earl's salts supplemented with 10% (v/v) fetal calf serum, 4 mM L-glutamine, 0.2 mM nonessential amino acids, and 1 mM sodium pyruvate. SMS-KAN cells (neuroblastoma, hY₂) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM L-glutamine, and 0.2 mM nonessential amino acids. BHK cells stably transfected with rY₅-receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 0.05% geneticin. Cells were grown to confluency at 37°C and 5% CO₂.

Binding assays

For binding assays at Y₁-, Y₂-, and Y₅ receptors, cells were resuspended in incubation buffer MEM with Earl's salts containing 0.1% bacitracin, 50 mM Pefabloc, and 1% bovine serum albumin. Two hundred microliters of the suspension, containing ~3.0 million cells per milliliter, were incubated with 25 μL of a 10-nM solution of ³H-propionyl-NPY and 25 μL of NPY or analog in a concentration range of 100 μM to 10 pM. Nonspecific binding was defined in the presence of 1 μM unlabelled NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation

at 1600g and 4°C for 5 min. The pellets were washed once with 400 μL phosphate buffered saline (PBS), centrifuged again, and resuspended in 100 μL PBS. The cell suspension was mixed with 3 mL scintillation cocktail and radioactivity was measured by a β^- counter.

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