# 行政院國家科學委員會專題研究計畫 成果報告

## 利用核磁共振方法探討合成的神經 peptide 片段在溶液及擬細胞膜環境中結構的變化(第 2 年)
## 研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2113-M-032-010-MY2
執 行 期 間 ：96 年 08 月 01 日至 97 年 07 月 31 日
執 行 單 位 ：淡江大學化學系

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處 理 方 式 ：本計畫可公開查詢

中 華 民 國 97 年 12 月 30 日

# 行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

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Introduction

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. A ligand, which has been made the object of many pharmacological and structural studies in the last years, is NPY [1], a member of neuropeptide Y (NPY) family [2]. The family of neuroendocrinic peptides includes the neurotransmitter NPY and peptide YY (PYY) [3, 4] and pancreatic polypeptide (PP) [5]. Each of these peptides contains 36 residues, characterized by a large number of tyrosine residues and amidated at its C-terminal end. They activate at least six the rhodopsin-like super-family of the GPCRs (G-protein coupled receptor) named Y1, Y2 ,Y3, Y4,Y5 , and y6 receptors [2]. Upon activation they inhibit the adenylate cyclase and lead to decreased levels of intracellular calcium.

NPY was first isolated from porcine brain based on its physicochemical properties [1]. It is the most abundant neuropeptide in the central nervous system of mammalians [6,7] and important for the regulation of food intake and is involved in the regulation of various physiological processes [8, 9]. PYY is predominantly synthesized in the digestive tract [10], and PP is expressed in the pancreas [11] and in gastrointestinal endocrine cells [12], from which they are released into the circulation after ingestion of a meal [13]. It promotes food intake and gastric emptying by activation of the $Y_4$ and probably $Y_5$ receptors [14, 15] in the central nervous system. Following list the peptide sequences of hNPY, which was studied in this project.

hNPY YPSKPDNPGE DAPAEDMARY YSALRHYINL ITRQRY-NH2

The first structure for a member of NPY family was the crystal structure of avian pancreatic polypeptide (aPP) [16]. The solution structure of bovine pancreatic polypeptide (bPP) has been determined by NMR in water [17]. Both aPP and bPP share a similar conformation referred to as the PP-fold model. The N-terminal part of the molecule (residues 1 − 8) adopts a polyproline type II helical conformation followed by a tight hairpin (residues 9 − 13) and fold back onto a well defined α-helix encompassing residues 14–31. A hydrophobic interaction between the helix and N terminal region maintained the packing between these two segment sequences. aPP in the crystal state has been reported to forms a symmetric dimmer and suggested that unligated bPP in solution also exists in the form of a dimer [18].

On the basis of the high homology between the primary structures of NPY and PP, the PP-fold was originally also postulated for hNPY [19]. However, structure studies in solution revealed that NPY forms a dimer dependent from its concentration and there are characteristically different structures existing between PP and NPY. The dimerization interface was postulated to comprise with antiparallel packing with hydrophobic interaction of two helical units that belong to two

different molecules. As the concentration is lowered, the monomer is formed with a smaller helical content in NPY [20]. Various structural studies indicate that the N-terminal residues play an interesting and sometimes unexplained role in the function of the peptide. Some authors have claimed that the N-terminal region is aligned antiparallel to the a helix (PP-fold) [19, 21]; other authors have claimed that it is unordered [22-25], forming a polyproline II helix [26] or a sequence of β turns [27]. A recently report has suggested that the back-folding of the PP-fold is against for NPY monomer in solution in which the N terminus is remained free and flexible [32].

The multiplicity of physiological processes in which NPY is involved owing to its binding to at least 6 G-protein coupled receptors (GPCRs). They are termed Y1, Y2, Y3, Y4, Y5, and y6. The Y1, Y2, and Y5 receptors bind preferentially to NPY and PYY, while Y4 is selective for PP. The structure of the free ligand is changed upon binding to the receptor so that the peptide fits the structural requirements of the binding sites. The structure and the dynamics of pNPY bound to DPC micelles in solution were investigated by Bader [28] that the N-terminal segment (residues 1–13) does not show any secondary structure while a -helix is proposed in residues 14–36. The axis of the α-helix is parallel to the membrane surface and the side of the amphipatic α -helix is involved in the interaction with the micelle surface by the hydrophobic face.

The C-terminal pentapeptide is important for functional activity and all investigations have emphasized on its functional role. Its structural role, that is say, how it affect the active conformation, still sparse reports present. In physiological concentration, monomeric form is ascendant with a less ordered structure which is defined as “dynamic state” [16] and seems not to guide receptor selection. At higher concentration monomer presents in minor percentage in equilibrium with the predominant dimmer. The hydrophobic face of the α-helix used for dimerization interface is also used for membrane binding. 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 -helical in the membrane-bound molecule. Evidence has confirmed the importance of NPY binding to cellular membranes as a fundamental step that induces the required conformation before the binding to the receptor. Based on these evidences we assume that the induced helix may affect the formation of a stable well-defined conformation and also the peptide self-associate state. To probe this problem we synthesized a series of short peptide fragments of hNPY with or without the C-terminal pentapeptide. The structural difference directly related to the peptide’s associate state may be monitored by pulsed-field-gradient (PFG) spin echo NMR experiment In this study we monitored the temperature dependent self-association of a NPY fragment with PFG-NMR experiment and determine the solution structures with NOE restrained dynamic method. The differences between these structures may point to the direction related to the dependence of peptide conformation on the C-terminal helix.

Experimental methods

*Peptide synthesis and purificaion*

A series of peptide fragments, hNPY[21-31], hNPY[20-36], hNPY[18-26], hNPY[18-26], and hNPY[15-29], cover the whole a-sequence of hNPY were synthesized (0.1mmol scale) by the solid phase methodology on a Rink amide 4-methylbenzhydrylamine resin using peptide synthesizer and the standard Fmoc procedure [29]. The synthetic peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a C18 column using a linear gradient (10–50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) in water, at a flow rate of 10 ml/min.

*NMR spectroscopy*

All NMR experiments were recorded with AVANCE 600NMR spectrometer (Bruker) equipped with a triple resonance probe including shielded *z*-gradients. Two 0.5 ml NMR samples were used: a 3mM aqueous sample (pH 3.5) containing 10% D2O, and a 2 mM TFE(50%TFE-D3/50%D2O) sample. For 2D experiments, temperature was set at 283K and 310 K in TFE. The following conventional two-dimensional experiments were carried out: 2D COSY, TOCSY, NOESY, 1H–13C HSQC [8]. TOCSY spectra were acquired with a 80 ms spin-lock time using the TOWNY composite pulse cycle in methanol and a MLEV-17 sequence in water. NOESY spectra were recorded with mixing times 200 ms. TOCSY, NOESY and HSQC experiments were performed in the phase-sensitive mode, using the time proportional phase incrementation method for quadrature detection (TPPI or States-TPPI). For the TOCSY and NOESY experiments, water suppression was achieved using the pulsed-field gradient-based watergate method [60]. For the heteronuclear experiments performed in aqueous solution and the COSY experiments, water resonance was suppressed with a low-power presaturation.

*PFG-NMR spectroscopy*

Pulsed field gradient spin echo (PGSE) experiments were performed with a pulsed field gradient unit producing a magnetic field gradient in the *z*-direction with a strength of 55.4Gcm−1. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used. The strength of the sine-shaped gradient pulse with a duration of 1.4 ms, was linear incremented in 32 steps, from 2% up to 95% of the maximum gradient strength, with a diffusion time of 200ms and a longitudinal eddy current delay of 25ms. 32 scans was recorded in 2D mode for each measurement, with a recycle time of 6 s between scans. To ensure that equilibrium had been established with respect to sample temperature and molecular self-association processes. After Fourier transformation and a baseline correction, the diffusion dimension was processed using the

DOSY subroutine of the Bruker Topspin software package.

## Results and discussion

5 peptides hNPY[21-31], hNPY[20-36], hNPY[18-26], hNPY[18-26], and hNPY[15-29] have be synthesized and purified. In this report, we will focus our discussion on hNPY[21-31] and hNPY[20-36] both peptides. A typical chromatogram of peptide hNPY[21-31] is shown in Fig. 1 with a main peak at retention time 15.063. Fig.2 shows the mass spectrum of the main peak in Fig. 2. The molecular mass of the synthesized peptide was verified to be 1361 as our expectancy.

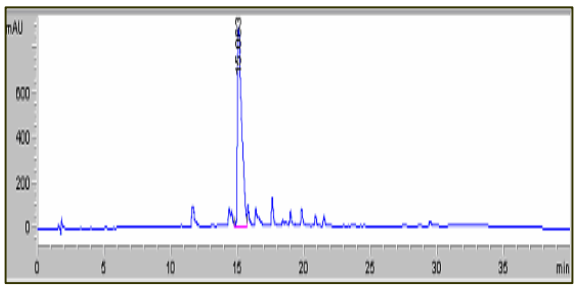


Fig. 1 The HPLC chromatogram of peptide hNPY[21-31] synthesized by SPPS indicate a main peak with a retention time about 15 minutes.

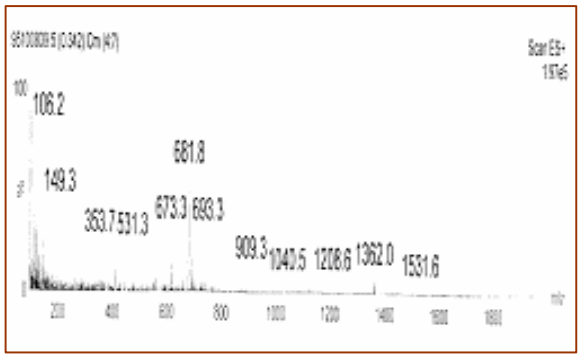


Fig. 2 The mass spectra of the collected main peak in Fig1 demonstrate the molecular mass is 1361, same as the peptide to be synthesized

CD measurements for all five peptides were carried out on a Jasco 700 spectrometer in trifluoroethanol/H20 over 180 - 250 nm. CD spectra of hNPY[21-31] in different concentration of TFE/water is shown in Fig 3 indicated that conformations of peptide fragments are TFE/water concentration dependent. The helix is most populated in 50% TFE/ 50%H2O for all peptide.

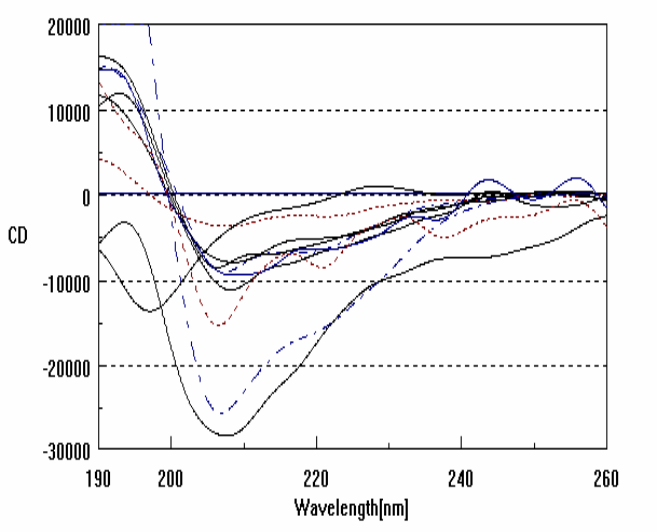


Fig. 3 The CD spectra of hNPY[21-31] were measured in different concentrations of TFE/water.

Part region TOCSY spectrum of hNPY[21-31] and the assignments are shown in Fig 4.

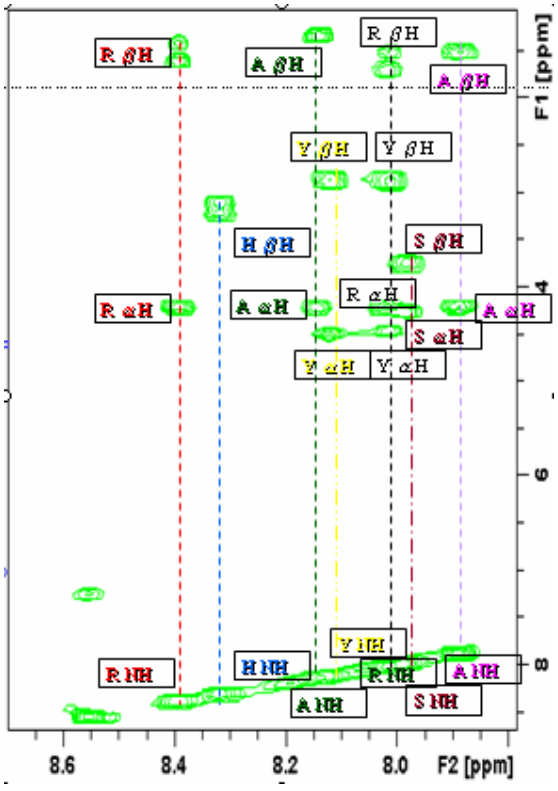


Fig 4. Part region of a TOCSY spectrum shows the coupling within a residue. Cross peak assignments of each amino acid spin system is labeled.

*Structure in water*

The 1H resonances of both peptide fragments in water were determined by a series of COSY, TOCSY and NOESY experiments. The 13Cα signals were assigned using 13C-1H HSQC experiments on a 13C natural abundance sample. However, two-dimensional NOESY spectra did not indicate any amide–amide connectivities in the amide region indicating that these peptides undergo conformational exchange in water and do not exhibit a stable secondary structure in water.

*Self-associate state in TFE*

Fig. 5 shows the plot of diffusion coefficients vs. temperature as a non-linear relationship due to some factors affecting diffusion such as viscosity and apparent molecular weight are also temperature dependent. It is plausible to assume viscosity effect is similar for both hNPY[21-31] and hNPY[20-36] peptide solutions. Since the viscosity and apparent molecular mass affect the slope in the same direction, the more deviated from linear relationships, therefore, implied the apparent molecular weight of hNPY[21-31] being more temperature dependent.

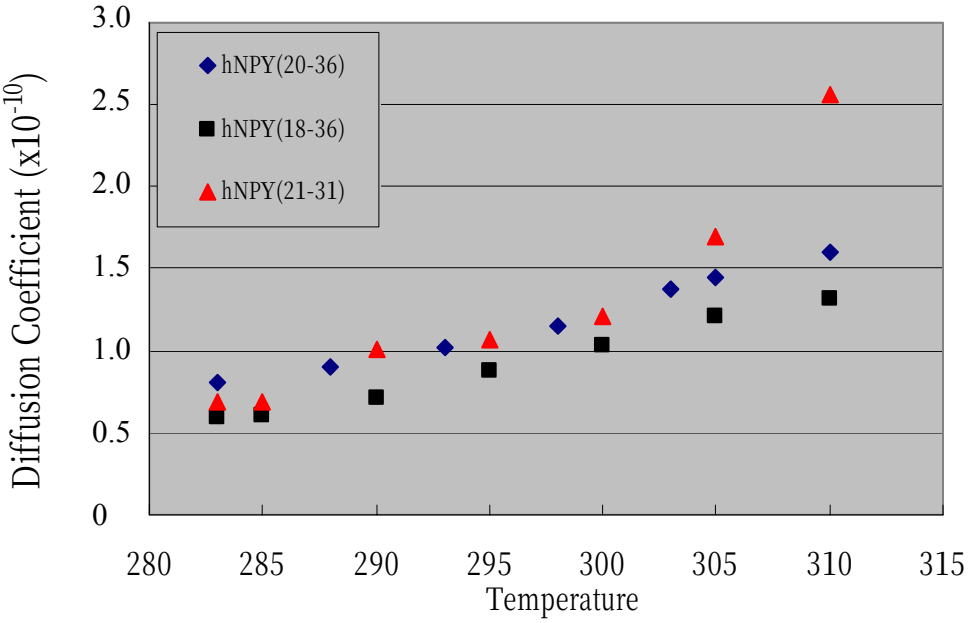


Fig. 5 Plot of diffusion coefficients vs. temperature of peptide fragments. Slope increase at higher temperature is more significant for hNPY[21-31] than the other two peptides implies the more temperature dependent apparent molecular weight if assume similar viscosity effect.

To simplify the calculation of apparent molecular weight at each temperature, we use TSP as an internal reference for viscosity measurement. Equation ($\eta=1.000+2.133x+1.670x^2-8.404x^3+4.984x^4$) was firstly used to estimate the solvent viscosity η (x donates the volume fraction of TFE per total volume of added TFE and water) at $20^0$C, which was then used to calculate the hydrodynamic radius of TSP by using Stokes-Einstein equation. This radius value was then used to calculate viscosity at each temperature. The apparent molecular weights then can be calculated with equation 1.

$$MD= (kT/6\pi\eta FD)^3[4\pi N_A/[3(\upsilon 2+\delta 1\upsilon 1)]]$$

where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the solution, $N_A$ is Avogadro's number, ν2 and ν1 are the partial specific volumes of the molecule and solvent water, respectively, and δ1 is the fractional amount of water bound to the molecule (hydration number)

To validate the calculation of apparent molecular weight, the other short peptide, hpp[17-24], was synthesized. This peptide was shown to be a random coil structure in water and water/TFE solution and not possible to form a dimmer. The calculated results ranged from 0.9 to 1.2 times of molecular weight indicated a monomer form and mostly not dependent upon temperature.

As shown in Fig 5, the apparent molecular weights calculated from diffusion data indicate equilibrium constant of peptide self-associate are quite temperature independent for both hNPY[20-36] and hNPY[18-36]. The enthalpies at both dissociate and associate states are therefore suggested being quite similar for hNPY[20-36] and hNPY[18-36] , that implies a stable self-associate conformation is existed through the alternated temperature range. In contrast, the equilibrium position shifted toward associate state with temperature decrease for hNPY[21-31] and hNPY[15-29]. The more temperature dependent equilibrium constant means the more enthalpy difference between both dissociate and associate states and multiple conformations are coexisted and changed from temperature to temperature.

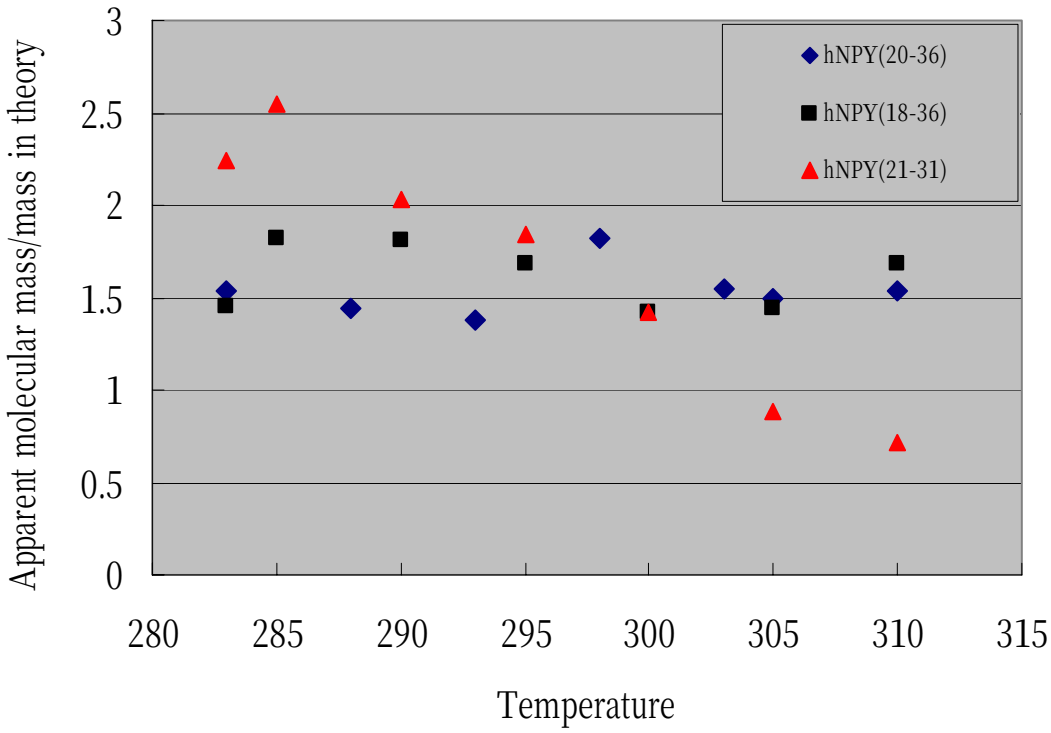


Fig. 5 Plot of the apparent molecular mass relative to the molecular mass in theory indicates the temperature dependence of associate-dissociate equilibrium position.

The enthalpy differences among these peptides may origin from the C-terminal residues forming a helix that may be an important factor for conformation stabilization. The peptide conformation with induced helix is enthalpy favorable and conformation responsible for membrane binding and recognized by receptor.

As the temperature decrease the apparent molecular weight of hNPY[21-31] increase, indicated the equilibrium shift toward the self-association. While in hNPY[20-36], apparent molecular weight is almost not temperature dependence indicated a stable conformation exist through the measured temperature range. In curious about how structure of hNPY[21-31] change with temperature implied by PFG experiment, we sampled the data at 285 and 310K.

*Structu e of hNPY[21-31] in TFE*

Proton resonances of peptides in TFE at both 310K and 283K are assigned. TOCSY and COSY spectra were used to assign spin systems of most of the amino acid residues, and NOESY data

provided the sequential connections between these spin systems according to the strategies of Wuthrich. A first assessment of secondary structural features was obtained by analysing 1Hα and 13Cα chemical shifts. The CαH protons chemical shift deviated from random coil CαH chemical shifts suggesting the possibility of peptide exhibiting a major stable conformation. The proton and carbon variations observed were inferior to 0.1 and 1 ppm, respectively, which reflects a predominantly random conformation in water. Residues 25–31 had upfield alpha proton and downfield carbon chemical shifts differing from the random coil values by more than −0.05 and 0.2 ppm, respectively, suggesting the presence of a helix between residues 25 and 31 induced by TFE. Sequential HN–HN cross-peaks observed in the NOESY spectrum were characteristic of helical regions. Indeed, the cross-peak pattern indicated the presence of a continuous α-helix. Besides, the observation of dNN($i$, $i$ + 1) sequential NOE cross-peaks as well as d_N($i$, $i$ + 3) suggested the possible existence of a nascent helix in peptides.

*Dimeric Structure in TFE at 283K*

Long range NOEs were observed for hNPY[21-31] at 285 K. Based on the diffusion data, we assigned these NOES as intermolecular connectivity due to contact between individual molecules in dimmer. To simulate the dimmer form, the starting structures were created by connecting two identical copies of the monomer helix by a chain of 16 glycines in a random conformation.

At 283K, residue 25-29 had deviation more than -0.2 ppm suggesting a shorter but more tight packing helix conformation. The cross-peak pattern indicated the presence of a continuous α-helix between residues 25 and 29. Besides, the observation of dNN($i$, $i$ + 1) sequential NOE cross-peaks as well as dαN($i$, $i$ + 3) also suggested the possible existence of a helix in this region of the peptide.

Conclusions

The qualitative thermodynamic point of view based on diffusion data may accounted for the observations from different laboratories. Since concentration-dependent conformation of NPY was suggested as equilibrium of dimeric aggregates with the monomeric neuropeptide Y, this clearly implied the structure is easily perturbed by alternation such as concentration, pH and temperature. The environment-sensitive conformations all have been reported with flexible C-terminal residue. In contrast, well defined not perturbed structures are reported with helix formation at C-termianl residues. The peptide conformation with induced helix therefore should be correlated with stabilized conformation which is responsible for membrane binding and recognized by receptor. We could successfully work in the NPY fragments at different temperatures. The differences between these structures point to the direction related to the dependence of peptide conformation on the C-terminal helix

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