Conformational Analysis of Neuropeptide Y-[18–36] Analogs in Hydrophobic Environments

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ABSTRACT The interactive and conformational behavior of a series of neuropeptide Y-[18–36] (NPY-[18–36]) analogs in hydrophobic environments have been investigated using reversed-phase high-performance liquid chromatography (RP-HPLC) and circular dichroism (CD) spectroscopy. The peptides studied comprised a series of 16 analogs of NPY-[18–36], each containing a single D-amino acid substitution. The influence of these single L → D substitutions on the α-helical conformation of the NPY-[18–36] analogs in different solvent environments was determined by CD spectroscopy. Retention parameters related to the hydrophobic contact area and the affinity of interaction were determined with an *n*-octadecyl (C_{18}) adsorbent. Structural transitions for all peptides were manifested as significant changes in the hydrophobic binding domain and surface affinity between 4°C and 37°C. The results indicated that the central region of NPY-[18–36] (residues 23–33) is important for maintenance of the α-helical conformation. Moreover, L → D amino acid residue substitutions within the N- and C-terminal regions, as well as Asn^{29} and Leu^{30}, do not appear to affect the secondary structure of the peptide. These studies demonstrate that RP-HPLC provides a powerful adjunct for investigations into the induction of stabilized secondary structure in peptides upon their interaction with hydrophobic surfaces.

INTRODUCTION

Neuropeptide Y, a 36-amino acid carboxy terminally amidated peptide, was first isolated from porcine brain in 1982 by Tatemoto. Various structure-function studies have demonstrated that the C-terminal fragment encompassing residues 18–36 of NPY (i.e., NPY-[18–36]) elicits a hypotensive response slower in onset and longer in duration than the hypertensive effect observed with NPY (Boublik et al., 1989, 1990). The ability of NPY-[18–36] to inhibit ^{125}I-NPY binding, with no effect on cardiac adenylate cyclase activity, suggests that NPY-[18–36] is a potent antagonist of NPY function (Balasubramaniam and Sheriff, 1990; Balasubramaniam et al., 1990). In addition, the structural requirements for NPY-[18–36]-induced hypotension, as seen from changes in mean arterial pressure (MAP), have been proposed to involve the N-terminus of NPY-[18–36] (Feinstein et al., 1992).

CD studies performed in 30% TFE in aqueous buffer have indicated that NPY-[18–36] adopts an α-helical structure that has been proposed to be essential for biological activity (Boublik et al., 1989; Spicer et al., 1990). It has also been proposed (Boublik et al., 1989) that an α-helical motif in the C-terminus of NPY-[18–36] is essential for biological activity, whereas Spicer et al. (1990) have shown that α-helicity at the N-terminus is not a strict requirement for biological activity. Edmundson helical wheel analysis (Aguilar et al., 1993) has revealed that NPY-[18–36] can adopt a strongly amphipathic helical structure. It has also been suggested that NPY-[18–36] adopts this helical conformation upon reaching the lipid-rich receptor environment (Boublik et al., 1989). The induction of helical structure within NPY-[18–36] as it interacts with lipid surfaces may therefore play an important role in receptor recognition. The ability to monitor the surface-induced stabilization of the secondary structure of NPY-[18–36] analogs with analogous hydrophobic surfaces represents an important approach to the characterization of the structure-function relationships of NPY-[18–36].

In a previous study from this laboratory, reversed-phase high-performance liquid chromatography (RP-HPLC) was employed to study the interaction with hydrophobic surfaces of the same series of NPY-[18–36] analogs with single D-amino acid substitutions (Aguilar et al., 1993). Significant changes in chromatographic parameters related to the contact area and affinity of interaction demonstrated that D-substitutions at residues Tyr^{27}-Thr^{32} severely disrupt the secondary structure of NPY-[18–36]. These chromatographic parameters were derived with the *n*-butyl (C_4)- and *n*-octyl (C_8)-silica adsorbents with acetonitrile or *i*-propanol as the organic modifiers. In the present study, the induction and stability of the proposed amphipathic α-helical structure of NPY-[18–36] and the NPY-[18–36] D-substituted analogs have been characterized with the more hydrophobic *n*-octadecylsilica (C_{18}) adsorbent. In addition, the propensity of these L → D substituted NPY-[18–36] analogs to adopt α-helical structures was also measured by CD and correlated with the reversed-phase retention behavior to provide further insight into the interactive conformation of these peptides at hydrophobic surfaces.

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MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (HPLC grade; MeCN) was obtained from Mallinckrodt (Paris, KY), and trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). *N*-Acetyl-L-phenylalanine ethyl ester, *N*-acetyl-L-tryptophanamide, and angiotensin I, II, and III were obtained from Sigma (St. Louis, MO). Porcine all-L-α-NPY-[18–36] and NPY-[18–36] D-substitution analogs, synthesized by manual solid-phase methods using t-Boc-protected α-amino acids, were generously provided by Dr. Jean Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute (La Jolla, CA) and Dr. Jaroslav Boublik (Baker Medical Research Institute, Prahran, Australia). The analogs included single D-amino acid substitutions at positions 18, 19, 20, 23, 25, 27, 28, 29, 30, 31, 32, 33, 34, 35, and 36, respectively. The sequences and molecular weights of all of the test solutes are listed in Table 1.

Circular dichroism analysis

Circular dichroism spectra for the all-L-α-NPY-[18–36] and the NPY-[18–36] D-substituted analogs were measured over the range 185–250 nm, using an AVIV model 62 DS CD spectrophotometer (AVIV Associates, Lakewood, NJ). All spectra were obtained at 25°C using cells with a pathlength of 1.0 mm. Data points were recorded at wavelength steps of 0.2 nm and a bandwidth of 0.8 nm. Five repeat scans were used to obtain the final averaged spectra. Multiple linear regression analysis was performed using the PLOT software package (AVIV). Reference spectra representing pure α-helix, β-sheet, β-turn, and random structures were used to determine the contribution of each structure to the overall CD spectra based on the algorithm of Provencher and Glöckner (1981). Solutes were dissolved in 15 mM orthophosphoric acid (P_i) buffer titrated with sodium hydroxide to pH 2.2, 40% acetonitrile (MeCN) (v/v) in 15 mM P_i buffer, and 90% 2,2,2-trifluoroethanol (TFE) (v/v) in 15 mM P_i buffer, and at concentrations of 25–200 μg/ml.

The wavelength dependence of the mean molar ellipticity, $[\theta]$ (deg cm^2 dmol^{-1}), was calculated from

$$[\theta] = (\mathrm{MRW} \times \theta)/C \times l,$$

where MRW is the mean amino acid residue molecular weight, C is the concentration (g cm^{-3}), l is the pathlength (cm), and θ is the ellipticity (°).

Chromatographic procedures

All chromatographic measurements were performed on a Hewlett-Packard (Walbronn, West Germany) HP1090M HPLC system equipped with a DR5 solvent delivery system, an automated injection and sampling system, an HP1090 diode-array detector (monitoring wavelength 214 nm) and a thermostatically controlled column compartment (temperatures 37–80°C). Low column temperatures (4–25°C) were controlled by immersing the column in a thermostated column water-jacket coupled to a recirculating cooler (FTS Systems, Stone Ridge, NY). Chromatographic analysis and peak integration were performed by an HP79994A Chemstation Analytical Workstation computer coupled to a ThinkJet printer.

Chromatography was performed with Bakerbond Analytical wide-pore *n*-octadecyl-silica (C_{18}) reversed-phase columns (J. T. Baker, Phillipsburg, NJ) with dimensions of 250 × 4.6 mm I.D., containing adsorbent of 5 μm nominal particle diameter and 30 nm average pore size. Bulk solvents were filtered under vacuum and degassed by sparging with helium. Linear gradient elution was performed using 0.1% TFA in water (buffer A) and 0.09% TFA in acetonitrile-water (50:50) (buffer B) over gradient times of 30, 45, 60, 90, and 120 min with a flow rate of 1 ml/min at temperatures of 25, 37, 60, and 80°C. The higher back pressures created at 4°C necessitated the use of a reduced flow rate of 0.8 ml/min at this temperature. Peptide solutions were prepared by dissolving the solute at concentrations of 0.2 mg/ml in 0.1% TFA in water and stored at −20°C until use. A 25-μl injection size was used for all chromatographic experiments. All data points were derived from duplicate measurements with retention times between replicates varying by less than 1%. The column dead volume (V_0) was taken as the retention volume of the noninteractive solute, sodium nitrate. Chromatographic parameters were calculated using the Pek-n-ese program (Aguilar et al., 1985) written in Pascal for an IBM PC, and statistical analysis involved ANOVA linear regression analysis.

RESULTS AND DISCUSSION

Circular dichroism spectroscopy

The Edmundson helical wheel representation of NPY-[18–36], shown in Fig. 1 *A*, clearly illustrates the propensity of this peptide to adopt an amphipathic α-helical structure. In Fig. 1 *A* the hydrophobic residues are indicated by bold black letters. The amino acid hydrophobicity classification was based on coefficients derived by Wilce et al. (1991, 1995) with C_{18}-silica adsorbents using acetonitrile as the organic modifier. The hydrophobic moment (Eisenberg et al., 1982), 0.72, was calculated for NPY-[18–36] using these retention coefficients. ^{1}H-NMR analysis of NPY-[18–36] in TFE/H_2O (9:1) (Mierke et al., 1992) has revealed that residues 19–34 can adopt a helical structure. The hydrophobic moment was calculated to be 0.97 for residues 19–34 of NPY-[18–36], suggesting that the amphipathic nature of NPY-[18–36] is predominantly associated with the central region. This amphipathic α-helical structure of NPY-[18–36] is illustrated in the lateral presentation in Fig. 1 *B*, where the hydrophobic moment is orientated downward. The amino acid residues (indicated by the *filled circles*), located in the lower face of the α-helix, will thus constitute the hydrophobic binding domain of NPY-[18–36].

To confirm the propensity of NPY-[18–36] to adopt an α-helical conformation in hydrophobic environments, the CD spectra were recorded in P_i buffer, 40% MeCN in P_i buffer, and 90% TFE in P_i buffer; the results are shown in Fig. 2. Although 90% TFE is commonly used to induce

TABLE 1 Sequences and molecular weights of solutes

Solute	Sequence	Molecular weight
N-Acetyl-L-phenylalanine ethyl ester (STD 1)	*N*-Ac-F-OEt	235
N-Acetyl-L-tryptophanamide (STD 2)	*N*-Ac-W-NH_2	245
Angiotensin I (ANGIO I)	DRVYIHPFHL	1297
Angiotensin II (ANGIO II)	DRVYIHPF	1046
Angiotensin III (ANGIO III)	RVYIHPF	931
NPY-[18–36]	ARYYSALRHYINLITRQRY-NH_2	2090

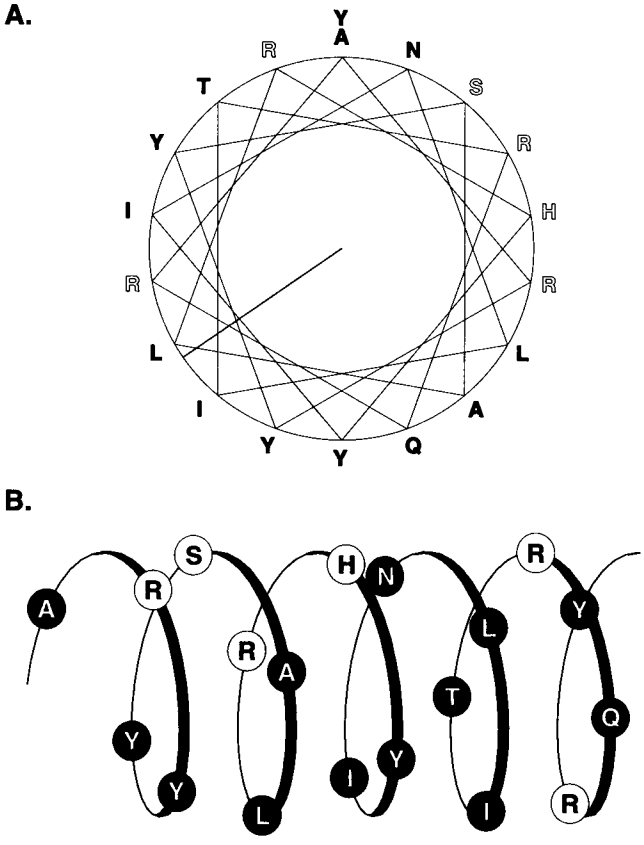

FIGURE 1 (*A*) Edmundson helical wheel representation of NPY-[18–36] demonstrating the amphipathic nature of the peptide. Hydrophobic residues are indicated by bold black letters and the direction of the hydrophobic dipole moment is indicated by the line from the center. (*B*) Lateral representation of NPY-[18–36] showing the putative helical structure of the peptide defined by the downward direction of the hydrophobic moment. Hydrophobic residues are indicated by the filled circles.

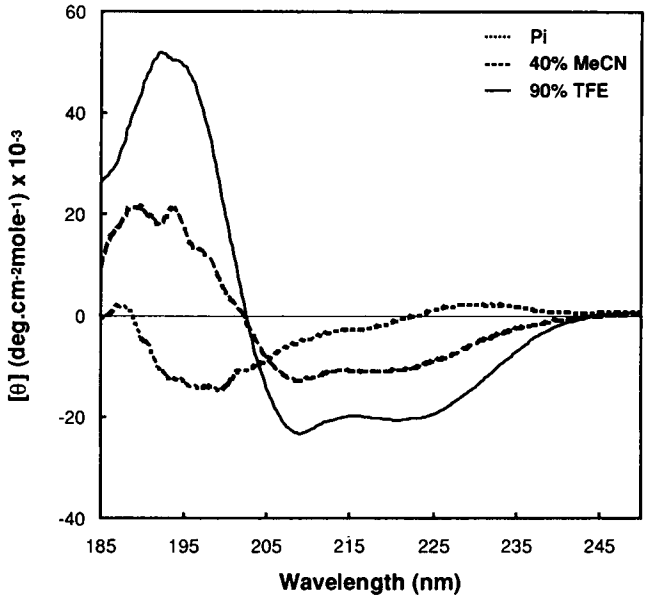

FIGURE 2 Circular dichroism spectra of NPY-[18–36] measured in 15 mM organophosphate (P_i) buffer, 40% acetonitrile (MeCN) in P_i buffer, and 90% 2,2,2-trifluoroethanol (TFE) in P_i buffer.

helical structure, 40% MeCN was employed in this study to mimic the solvent conditions that the peptide experiences when eluted from the C_{18} adsorbent. As evident from the results, NPY-[18–36] did not adopt any helical structure in P_i buffer. However, the α-helical content increased to 34.4% in 40% MeCN in P_i buffer and to 74.4% in 90% TFE in P_i buffer. The mechanism by which organic solvents can induce the formation of secondary structure in peptides remains ill defined, but is thought to involve interaction of the peptide dipoles in specific preferred conformations with the solvent molecules, thus leading to the stabilization of particular conformations over others through the participation of intramolecular hydrogen bonds and solvent-α-amide hydrogen bonding (Hodges et al., 1988; Storrs et al., 1992). The observed differences in α-helical content demonstrate that the conformation adopted by NPY-[18–36] in solution is strongly influenced by the solvent conditions. In particular, the degree to which acetonitrile and trifluoroethanol enhanced the intramolecular interactions within the peptide and stabilize the α-helical structure adopted by NPY-[18–36] differed significantly.

To assess the influence of the single D-amino acid substitutions on the α-helical content of NPY-[18–36], CD spectra were also recorded for each analog using identical buffer conditions. The percentage of α-helical structure in P_i buffer, 40% MeCN in P_i buffer, and 90% TFE in P_i buffer for each analog, plotted against the residue position of the D-amino acid substitution, is illustrated in Fig. 3. The results obtained in the aqueous P_i buffer demonstrate that all D-substituted peptides exhibit an essentially extended structure. However, the results obtained in 40% MeCN and 90% TFE for the NPY-[18–36] analogs reveal that all peptides can adopt a significant degree of α-helical structure in the presence of these organic solvents (Fig. 3). The variation in the α-helical content between the different NPY-[18–36] analogs is an indication of the degree of disruption caused by the chiral substitution. In 40% MeCN, the helical content of analogs with D-substitutions in the central region (residues 26–32) appear to be significantly affected, indicating that residues in this region are critical for the maintenance of secondary structure. In contrast, the α-helical structure of [D-Asn]^{29}-NPY-[18–36] was not significantly reduced compared to the corresponding L-α-analog, indicating that the D-isomeric substitution at this position does not significantly disrupt the overall α-helical conformation, i.e., chiral substitution of this residue is not critical for maintenance of the amphipathic α-helix. In 90% TFE, the reduced α-helical content exhibited by the analogs [D-Ala]^{23}- to [D-Arg]^{35}-NPY-[18–36] suggests that these residues are also located within the α-helical domain of NPY-[18–36]. Under these conditions, [D-Tyr]^{27}-NPY-[18–36] has the highest α-helical content, indicating that the L $\rightarrow$ D substitution at Tyr^{27} may enhance intrahelical hydrogen bonds in the hydropho-

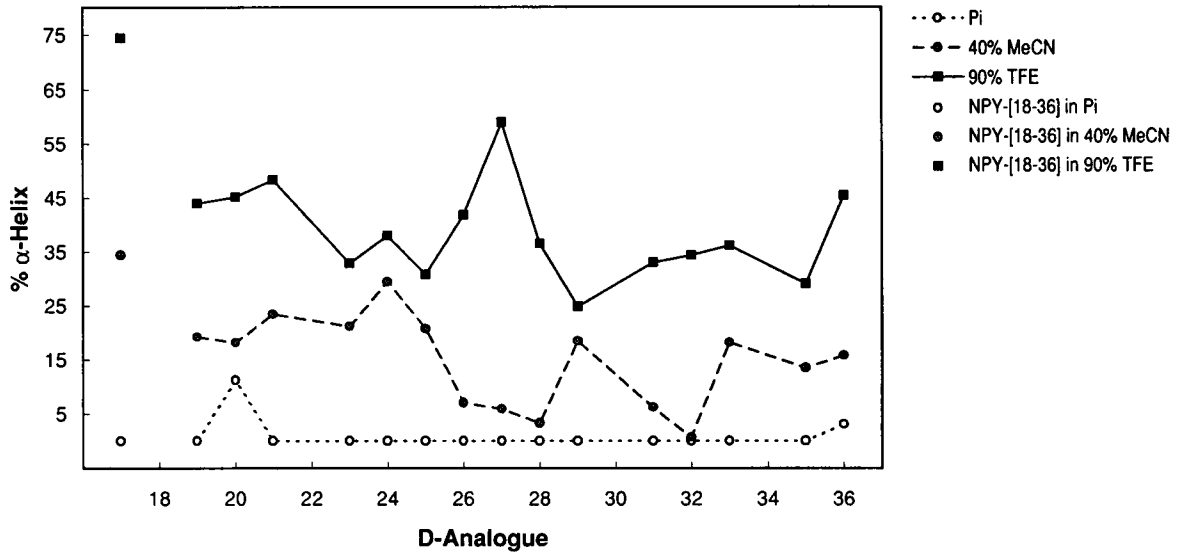

FIGURE 3 CD-determined α-helical content of the all-L-NPY-[18–36] peptide and the NPY-[18–36] D-substituted analogs in 15 mM organophosphate (P_i) buffer, 40% acetonitrile (MeCN) in P_i buffer, and 90% 2,2,2-trifluoroethanol (TFE) in P_i buffer.

bic environment provided by 90% TFE. The increased degree of secondary structure may be attributed to enhanced intramolecular interactions within the peptide, stabilizing the α-helical structure adopted by the peptides. Consequently, it can be proposed that the α-helix content of NPY-[18–36] can be increased according to the environmental conditions and that an α-helical structure is predominantly favored in the central region of the NPY-[18–36] peptides.

RETENTION RELATIONSHIPS

Dependence of log $\bar{k}$ on $\bar{\psi}$

Amphipathic helical structures are known to play an important role in many surface-mediated biorecognition phenomena. Recently, RP-HPLC has emerged as a particularly powerful technique for studying the influence of very subtle differences in amphipathic peptide structure upon interaction with hydrophobic ligands (Purcell et al., 1995; Blondelle et al., 1995b), as well as characterizing the folding/unfolding transitions of peptides and proteins at hydrophobic surfaces (Hodges et al., 1994). A variety of approaches have been employed to evaluate the changes in the reversed-phase retention behavior of amphipathic peptide and globular proteins, with the linear solvent strength model frequently employed to provide information associated with the interactive properties of peptide and protein solutes when linear gradient elution conditions are used (Stadalius et al., 1984; Aguilar et al., 1985; Purcell et al., 1989). A linear relationship is often observed in the plots of the median capacity factor, log $\bar{k}$, versus the fraction of organic solvent, $\bar{\psi}$, required to desorb the peptide or globular protein from the immobilized hydrophobic *n*-alkyl ligands, i.e.,

$$\log \bar{k} = \log k_0 - S\bar{\psi}, \quad (1)$$

where S is the slope and $\log k_0$ is the y intercept. Solvophobic theory has been used to derive a relationship between $\log \bar{k}$ and a number of physicochemical properties (Horváth et al., 1976; Snyder, 1980; Melander et al., 1984; Purcell et al., 1992; Aguilar et al., 1993, 1994), as follows:

$$\log \bar{k} = \log k_0 - (N\Delta A_h + 4.836N^{1/3}(\kappa^e - 1)V^{2/3})/RT, \quad (2)$$

where ΔA_h is the relative hydrophobic contact area, N is Avogadro's number, V is the mean molar volume of the solvent, R is the gas constant, T is the absolute temperature, and κ is the ratio of the energy required for the formation of a cavity with the surface area equal to the solute surface area and the energy required to extend the planar surface of the liquid by the same area (Purcell et al., 1992). As documented elsewhere (Aguilar et al., 1994), rearrangement of Eqs. 1 and 2 yields the following relationship between S and the solvophobic parameters, for a defined temperature and range of $\bar{\psi}$:

$$S = a\Delta A_h + b\kappa^e - c, \quad (3)$$

where a, b, and c are constants. The S value can thus be related to the hydrophobic contact area as derived from the solvophobic theory, and the $\log k_0$ value can be related to the affinity of interaction of the solute for the nonpolar ligands. Changes in the magnitude of the S and $\log k_0$ parameters as a function of temperature therefore represent important probes for variations in the secondary structure of polypeptides during interaction with the hydrophobic surfaces. The propensity of the NPY-[18–36] analogs to adopt preferred conformations at hydrophobic surfaces within the

context of the reversed-phase environment was consequently examined in terms of the dependence of S and log k_0 versus temperature.

In these experiments, retention measurements were obtained for all peptides on a C_{18} adsorbent for gradient times of 30, 45, 60, 90, and 120 min, at a flow rate of 1 ml/min, and temperatures of 4, 25, 37, 60, and 80°C, with linear gradients from water containing 0.1% TFA to water-acetonitrile (50:50) containing 0.09% TFA. Fig. 4, *A* and *B*, illustrates the dependence of log $\bar{k}$ on $\bar{\psi}$ for NPY-[18–36] and the NPY-[18–36] analogs chromatographed on C_{18}-silica at 25°C. If all of the NPY-[18–36] analogs interacted with the immobilized C_{18} ligands without any preferred orientation or conformation, then each D-substitution should exert the same relative influence on the retention times, and the retention plots of log $\bar{k}$ versus $\bar{\psi}$ for all analogs should be superimposable. However, the retention behavior of the NPY-[18–36] analogs, shown in Fig. 4, indicates that significant resolution was observed between a number of analogs, as has been recognized previously with other peptide diastereomers (Aguilar et al., 1993; Krausse et al., 1995). This is also evident from the representative chromatograms in Fig. 5 of the all-L-NPY-[18–36] peptide with the NPY-[18–36] analogs with D-substitutions at positions 19, 20, 23, 25, and 28 obtained at 4°C with a gradient time of 60 min. These results demonstrate that the interactive behavior of the different peptides is particularly sensitive to D-amino acid substitutions. In addition, the retention time of the all-L-NPY-[18–36] peptide and each D-substituted analog is plotted in Fig. 6. Relative to the parent all-L-α-NPY-[18–36], a general trend in the elution behavior of the peptides was also apparent. The analogs with D-substitutions in the N- and C-terminal regions (e.g., residues 18, 19, and 33–36) were generally characterized by longer retention times than the all L-α-NPY-[18–36] peptide, indicating that the hydrophobic contact region has increased. In addition, [D-Asn]^{29}- and [D-Leu]^{30}-NPY-[18–36] exhibited retention times similar to that of L-α-NPY-[18–36], suggesting that the hydrophobic contact regions of these peptides were unaffected by

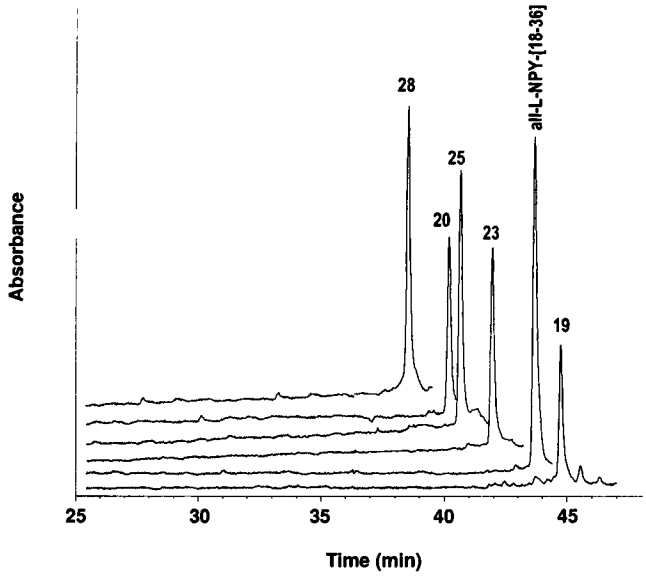

FIGURE 5 Representative chromatograms showing the separation of the all-L-NPY-[18–36] peptide and the NPY-[18–36] analogs with D-substitutions at positions 19, 20, 23, 25, and 28 chromatographed on C_{18}-silica at a temperature of 4°C with 50% acetonitrile as the organic modifier and using a 60-min gradient time. For clarity, the chromatograms have been vertically displaced.

D-substitutions at these positions. However, D-substitution of the central-region amino acid residues (20–28 and 31–32) significantly affected the peptide-ligand interaction and resulted in earlier elution times. For example, [D-Ile]^{28}-NPY-[18–36] exhibited the smallest retention time of all the analogs, indicating that the chromatographic contact region of this peptide had been significantly affected as a result of this chiral substitution. Overall, the retention data for the NPY-[18–36] analogs demonstrate that single D-amino acid substitutions resulted in changes in retention time as a consequence of the variation in the magnitude and composition of the hydrophobic contact region established between the peptide and the immobilized hydrophobic C_{18}

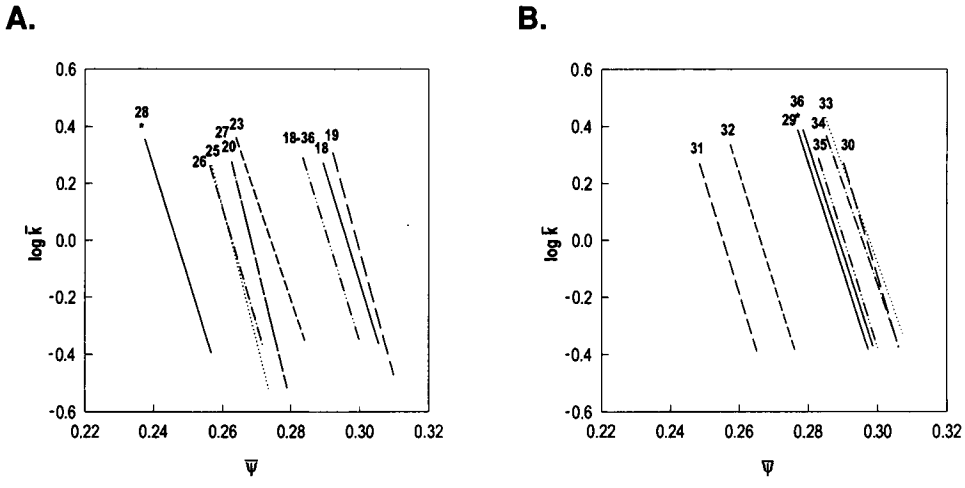

FIGURE 4 Dependence of log $\bar{k}$ on $\bar{\psi}$ for NPY-[18–36] and the NPY-[18–36] analogs chromatographed on C_{18}-silica at 25°C. The plots were derived from best-fit analysis to the data points (excluded for clarity) r = correlation coefficient (>0.95). Other chromatographic conditions are given in Materials and Methods. The retention plot for each D-substitution analog is designated by the residue position of the D-amino acid substitution. 18–36 = all-L-α-NPY-[18–36]. (*A*) Plots for the analog with substitutions in the N-teminal region of NPY-[18–36]; (*B*) plots for the analogs with substitutions in the C-terminal region.

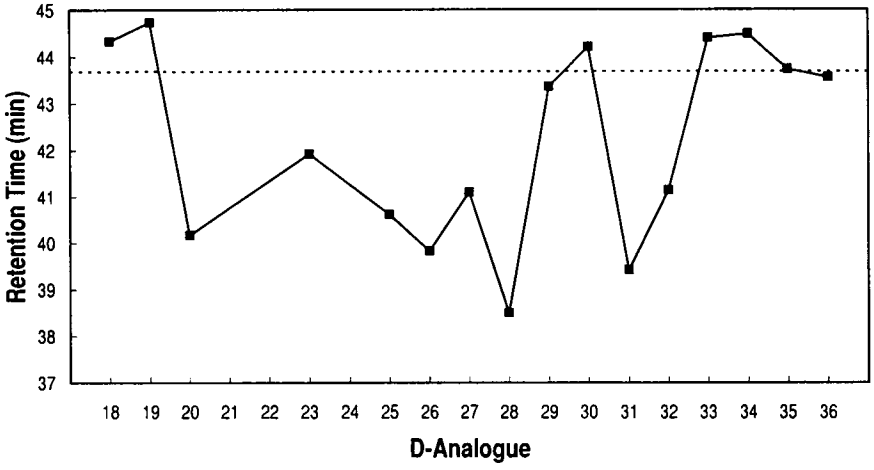

FIGURE 6 Dependence of retention time on D-amino acid substitution for the all-L-NPY-[18–36] peptide, indicated by the dotted line, and the NPY-[18–36] D-substituted analogs, indicated by the squares, chromatographed at 4°C, on C_{18}-silica, with 50% acetonitrile as the organic modifier, using a 60-min gradient time.

ligands. Similar conclusions can be drawn from the observations recently made by Kirby et al. (1993) and Krausse et al. (1995) with other amphipathic peptides after L → D substitutions.

These results also suggest that if the hydrophobic binding domain of each peptide analog is dependent on the extent of secondary structure (i.e., the degree of α-helicity), then a direct relationship should exist between the percentage helicity determined from CD measurement and the variation in retention time for the different D-substituted NPY-[18–36] analogs. Fig. 7 shows the dependence of percentage helicity in both 40% MeCN and 90% TFE, for each analog as a function of retention time with a 120-min gradient at 25°C. It is evident from Fig. 7 that the α-helical content of the NPY-[18–36] D-substitution analogs determined in 40% MeCN correlates well with retention times of the analogs with a correlation value of $r = 0.60$, significant at a 95% confidence level. In contrast, no correlation was observed between the α-helical content in 90% TFE and the retention times of the analogs chromatographed on the C_{18}-silica. This result would be expected, as the mechanism by which TFE and MeCN induce and stabilize peptide secondary structure will differ, particularly as a result of the different hydrogen bonding properties of these two organic solvents. More specifically, it is evident from these results that the hydrophobic binding domain located within the α-helical structure, as depicted in Fig. 1 *B*, is disrupted by the D-substitution of certain amino acids as a result of perturbation of the α-helical structure. Although the CD data analysis of the secondary structures of the NPY-[18–36] analogs provides insight into the conformations adopted by these analogs in solution, the CD data do not take into consideration the induction of secondary structure upon interaction with the C_{18} ligands under the RP-HPLC conditions. The influence of temperature on the retention parameters related to peptide structure was consequently examined.

Dependence of S and log k_0 values on temperature

The retention behavior of peptides can be readily investigated using RP-HPLC under conditions designed to alter the peptide structure. Previous studies with different classes of peptides and proteins (Richards et al., 1994; Purcell et al., 1995) have demonstrated that significant transitions in the

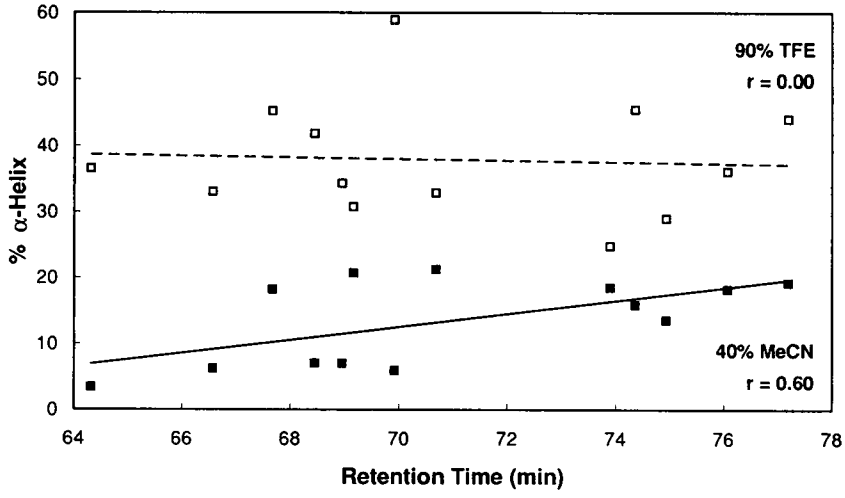

FIGURE 7 Correlation of α-helical content, determined by CD analysis in 40% acetonitrile (MeCN) and 90% 2,2,2-trifluoroethanol (TFE), with the retention times of the NPY-[18–36] analogs chromatographed on C_{18}-silica with 50% acetonitrile as the mobile phase at 25°C with a 120-min gradient time. Correlation coefficients (r) are indicated for each solvent condition.

magnitude of the S and log k_0 parameters occur with increasing temperature, as a result of perturbation of the secondary or tertiary structure of the solute. In the present study, S and log k_0 values for each NPY-[18–36] analog were evaluated as a function of temperature from regression analysis of plots of log $\bar{k}$ versus $\bar{\psi}$, as previously described (Purcell et al., 1989). In particular, the gradient elution behavior of NPY-[18–36] and the NPY-[18–36] D-substitution analogs were determined with the C_{18} adsorbent at temperatures of 4, 25, 37, 60, and 80°C. In addition, two amino acid derivatives, *N*-acetyl-L-phenylalanine ethyl ester (STD 1) and *N*-acetyl-L-tryptophanamide (STD 2), and the peptides, angiotensin I, II, and III (ANGIO I, II, and III), were also investigated as control solutes under the same experimental conditions. The sequences and molecular weights of these control solutes are listed in Table 1. Because no secondary structure is adopted by these amino acid derivatives, and as the angiotensin peptides possess minimal secondary structure under the experimental conditions, changes in retention behavior of these control solutes will not be related to conformational changes in the interactive structure, as documented in previous studies (Aguilar et al., 1993; Purcell et al., 1995). The dependence of S and log k_0 values on temperature for the control solutes is shown in Figs. 8, *A* and *B*. Essentially constant S values and uniform decreases in log k_0 values were observed with increasing temperature, consistent with the known thermodynamic behavior of the interaction of these control solutes with *n*-alkyl ligands (Aguilar et al., 1994).

The dependence of the S and log k_0 values on temperature for L-α-NPY-[18–36] is also shown in Fig. 8, with a transition between 25 and 37°C. These changes in the S and log k_0 values are in sharp contrast to the relatively constant values observed for the control amino acid derivatives and angiotensin peptides. Collectively, these results suggest that the interactive nature of the hydrophobic binding region of L-α-NPY-[18–36], localized on one face of the proposed amphipathic helix shown in Fig. 1, is disrupted at temperatures between 25 and 37°C. Similar large changes in the S and log k_0 values of these NPY-[18–36] peptides with temperature have been previously observed using the C_4 and C_8 ligands (Aguilar et al., 1993). However, the shape of the transitions for NPY-[18–36] differed from the behavior observed in the present study with the C_{18} silica, a finding that illustrates the influence of the chain length of the hydrophobic ligand on the induction of peptide conformation.

The dependence of the S and log k_0 values on temperature for the NPY-[18–36] D-substituted analogs is shown in Fig. 9. At 4°C there were only small variations in these parameters for analogs with D-amino acid substitutions in the N-terminal region. However, large variations in the S and log k_0 values were observed for the analogs with D-amino acid substitutions in the C-terminal region (e.g., residues 23–33). These results in the *n*-octadecyl environment suggest that at low temperatures the all-L-α-NPY-[18–36] peptide can assume a significant degree of secondary structure which, from the CD studies, is likely to constitute an α-helical conformation. Examination of the helical wheel projections for this peptide shown in Fig. 1 suggests that the hydrophobic binding domain involves residues 20, 21, 23, 24, 27, 28, 30, 31, 32, 34, and 35. However, this secondary structure can be disrupted by D-substitutions in the central

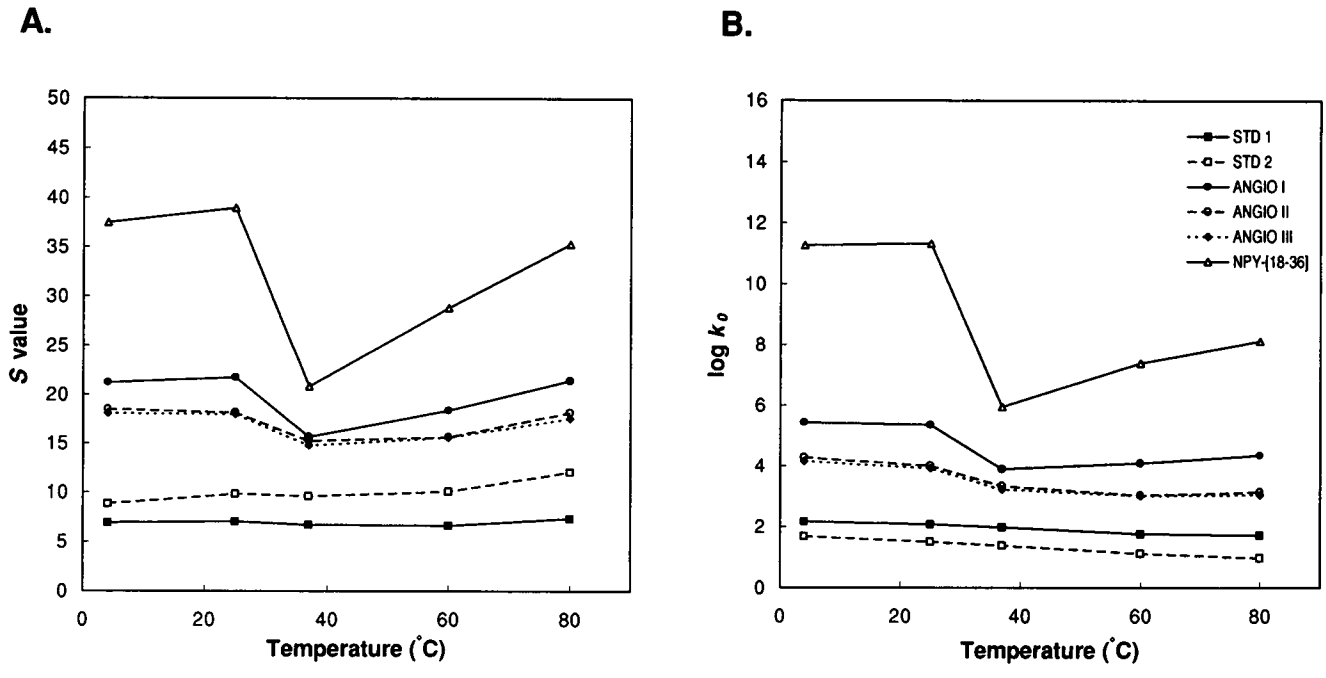

FIGURE 8 Dependence of S values (*A*) and log k_0 (*B*) on temperature for NPY-[18–36] and the control solutes, angiotensin I, II, and III (ANGIO I, II and III) and *N*-acetyl-L-phenylalanine ethyl ester (STD 1) and *N*-acetyl-L-tryptophanamide ethyl ester (STD 2) chromatographed on C_{18}-silica, with 50% acetonitrile as the organic modifier.

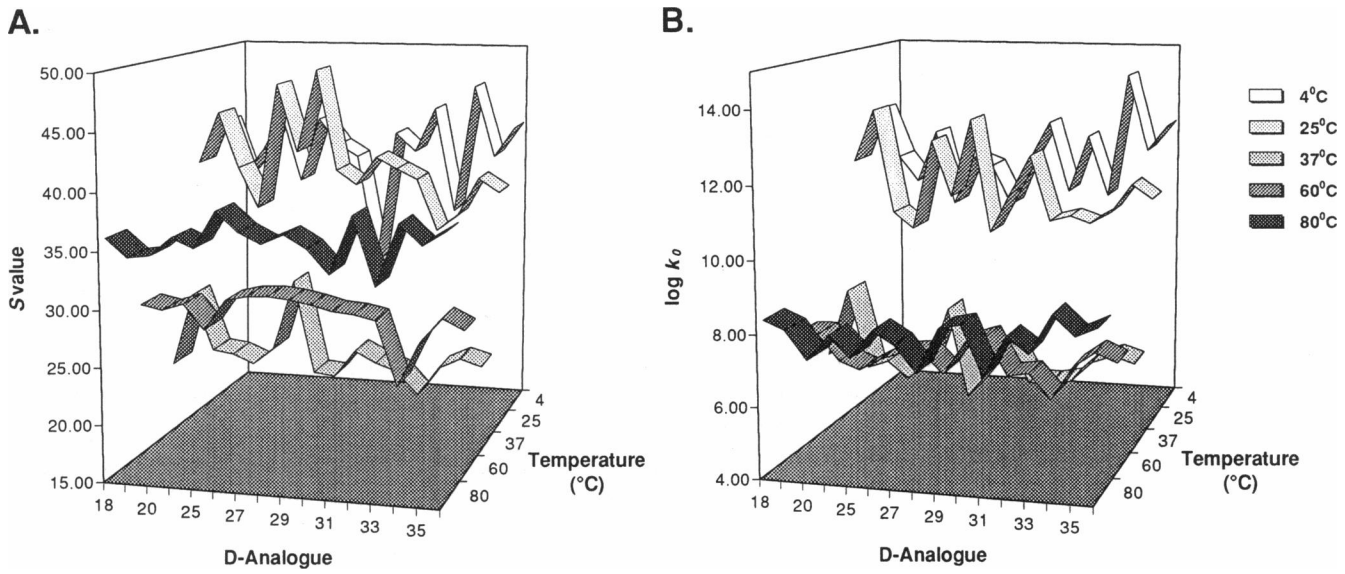

FIGURE 9 Dependence of S values (*A*) and log k_0 (*B*) on temperature for the NPY-[18–36] D-substituted analogs chromatographed on C_{18}-silica, with 50% acetonitrile as the organic modifier.

region of the peptide (residues 23–33). At 4°C, sharp decreases in the *S* values were observed for [D-Asn][29] and [D-Arg][33]-NPY-[18–36], suggesting that these residues are important for maintenance of the conformation adopted by NPY-[18–36] at this temperature. At 25°C, large variations in the *S* values were observed for NPY analogs containing D-substitutions between residues 23–28, with smaller changes apparent for the N- and C-terminally substituted analogs. The results from the CD studies indicate that a partial α-helical structure is maintained in the central region as D-amino acid substitutions between residues 23–28, and perturbation of this structure will cause large variations in the *S* and log k_0 values of these analogs as the temperature is increased. If the *S* value is a measure of the hydrophobic contact region, then a higher degree of α-helicity should result in high *S* values. However, there was no correlation between the percentage of helix in 40% MeCN and 90% TFE and *S* values at 4°C ($r = 0.48$ and 0.38, respectively; data not shown). This lack of correlation indicates that the *S* value is a measure of the surface-bound contact area, whereas the percentage helix values for each analog were determined in bulk solution and will not necessarily be directly related to the extent of secondary structure induced upon binding to the hydrophobic surface.

These results also suggest that the secondary structure that exists at 4°C becomes destabilized at 25°C, consistent with the diminished effect of C-terminal D-amino acid substitutions on the *S* and log k_0 values. In addition, the retention data obtained at 37°C demonstrated a significantly reduced hydrophobic contact region and affinity for all analogs compared to the data obtained at 4 and 25°C. With the exception of [D-Arg][19]- and [D-Tyr][27]-NPY-[18–36], which exhibited higher *S* and log k_0 values relative to NPY-[18–36], there was little variation in the magnitudes of these *S* and log k_0 values at 37°C. These results further demonstrate that the influence of D-substitution on the interactive structure of each analog has been significantly reduced between 25 and 37°C.

At 60°C, the *S* values increased relative to the data at 37°C and were generally constant for all of the analogs (except for [D-Arg][33]-NPY[18–36]), whereas the log k_0 values remained unchanged at this higher temperature. At 80°C, the *S* and log k_0 values increased again relative to the 60°C data, with small variations in the *S* and log k_0 values apparent for the analogs with D-amino acid substitutions in the C-terminus. Furthermore, the results indicate that at elevated temperatures, where the peptides are likely to adopt an extended conformation, D-substitution with the structurally larger amino acid residues, such as tyrosine and arginine, can still influence the chromatographic behavior of these peptides. This decrease at 37°C and the subsequent increases at 60 and 80°C in *S* and log k_0 values reflect the subtle variations in interactive structure and hence the nature of the amino acids that constitute the hydrophobic binding domain at each temperature.

CONCLUSIONS

In the present study, the combined use of RP-HPLC and CD measurements has been employed to investigate the induction and stabilization of peptide secondary structure at a hydrophobic surface. The results clearly demonstrated that the interactive conformation of NPY-[18–36] and the NPY-[18–36] D-substituted analogs at a hydrophobic surface is consistent with the induction of an amphipathic α-helical structure. Analysis of the retention parameters, *S* and log k_0, as a function of temperature has indicated that D-amino acid

substitutions in the central region (residues 23–33) of NPY-[18–36] caused the largest disruption in the conformational and interactive behavior. In contrast, residues at the N- and C-terminal regions as well as Asn^{29} and Leu^{30} appear to have a much smaller influence on the secondary structure of this peptide. Overall, these studies have further demonstrated the sensitivity of peptide retention behavior in RP-HPLC to small changes in peptide secondary structure. The ability of these procedures to provide structural and thermodynamic information on the interaction of peptides with hydrophobic surfaces has also been illustrated. The application of these procedures in conjunction with in situ spectroscopic techniques (Blondelle et al., 1995a) should offer considerable potential to probe the induction and stabilization of peptide conformation upon binding to lipid-like surfaces.

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