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High-performance liquid chromatography of amino acids, peptides and proteins

CXXVIII. Effect of D-amino acid substitutions on the reversed-phase high-performance liquid chromatography retention behaviour of neuropeptide Y[18–36] analogues

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ABSTRACT

The reversed-phase high-performance liquid chromatographic (RP-HPLC) gradient elution behaviour of a series of peptides related to Neuropeptide Y (NPY) has been investigated. The peptides studied included NPY, NPY[13–36], NPY[18–36] and a series of 16 analogues of NPY [18–36], each with a single D-amino acid substitution. Chromatographic parameters which relate to the interactive contact area and the binding affinity have been evaluated with two different stationary phase ligands and two organic modifiers. The results demonstrate that D-amino acid substitutions in the sequence region encompassing amino acid residues NPY [27–31] of these NPY [18–36] peptides significantly influence the interactive behaviour of these peptides relative to the unsubstituted NPY [18–36] molecule, while substitutions in the N- and C-terminal regions had little effect. Further, these results indicate that, in hydrophobic environments, NPY[18–36] adopts a significant degree of secondary structure which is severely disrupted by the presence of the D-amino acids in the central portion of the molecule.

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INTRODUCTION

The past ten years has seen an explosive growth in the use of the interactive modes of chromatography for the analysis and purification of peptides, proteins and polynucleotides. Nevertheless, the development of detailed theoretical and mechanistic descriptions of the interaction of biological macromolecules with chromatographic surfaces has still lagged behind the instrumental advances made in the field of chromatography. Recently however, the widespread practical application of liquid chromatography has prompted a variety of significant investigations into the molecular basis of the retention process with biomacromolecules [1–4]. As a consequence, high-performance chromatographic techniques are now finding use in the characterisation of the physico-chemical origin of the interactive phenomena involving peptides and proteins with biological or chemical surfaces and providing new insight into the dynamic behaviour of these biomacromolecules with a facility which cannot be easily replicated by other experimental methods. In particular, these techniques permit rapid assessment of the structural and molecular features of biopolymers which are responsible for their interactive behaviour at liquid–solid interfaces under a wide range of experimental conditions. Due to advances in sorbent development, the interactive surfaces and conformations of peptides and proteins can now be reliably probed through the analysis of chromatographic retention and bandbroadening behaviour. For example, the use of reversed-phase high-performance liquid chromatography (RP-HPLC) and hydrophobic interaction chromatography can provide useful insight into the mechanism of self association [5–7], the assembly of amphipathic helices [3,4,8,9] and the biorecognition behaviour of peptides and proteins in hydrophobic enviroments [10,11]. Similarly, high-performance size-exclusion chromatography (HPSEC) can be used to follow macroscopic processes associated with peptide or protein unfolding/refolding [12,13] and protein–protein interactions [14], while high-performance ion-exchange (HPIEC) can also be employed to fulfil similar objectives [15–17].

In the present study, the relative stability and interactive behaviour of a series of peptide analogues related to Neuropeptide Y (NPY) have been studied by RP-HPLC techniques. NPY is a polypeptide of 36 amino acid residues with an amidated carboxy terminus, first isolated from porcine brain in 1982 [18,19]. NPY is highly conserved between species, widely distributed in the central and peripheral nervous systems and possesses a multiplicity of physiological functions [20]. Since its discovery, NPY has been the focus of intense research aimed at establishing the molecular basis for its complex biological effects. The three-dimensional structure of NPY in the crystalline state has yet to be determined in detail. However, NPY shares a high degree of sequence homology with avian pancreatic polypeptide (aPP). The three dimensional structure of aPP, which has been determined by X-ray crystallography at 0.98 Å resolution, has therefore served as a model for the three dimensional structure of NPY [21]. A characteristic feature of aPP, and by inference of NPY, is the so-called PP-fold consisting of a polyproline-like helix comprising amino acid residues Tyr[1]–Pro[8] which lies parallel to an amphipathic α-helix comprising residues Glu[15]–Leu[30]. These two segments are linked by a type I β-turn from residues Gly[9]–Ala[12] and the overall structure is stabilised through hydrophobic interactions between the two helical segments. However, two-dimensional NMR studies of NPY performed in aqueous solution at pH 3.1 have indicated the existence of a long α-helical segment from amino acid residues Asp[11]–Tyr[36] with less well-defined structure at the N- and C-termini suggesting that the aPP-type fold does not exist in solution [22]. In contrast, the recent NMR determination of the solution structure of bovine pancreatic polypeptide (bPP) showed close similarity with the crystal structure of aPP [23].

NPY and PPs share common functional binding sites that can be subdivided into two receptor sub-types [24,25]. The importance of the PP-fold in NPY for receptor binding has been established since it has generally been observed that full binding activity for both types of NPY receptors requires the presence of both the N- and C-

terminal segments [24,26]. It has also been suggested that the PP-fold plays a role in determining the binding of the amphipathic α-helical segment of NPY to phospholipids thereby further modulating its biological activity [27,28]. Additionally, several structure–function studies have established that the C-terminal fragment of NPY encompassing residues Ala[18]–Tyr[36], which includes the sequence region of the putative amphipathic α-helix, retains significant hypertensive activity [29–31].

The interactive behaviour with hydrophobic surfaces of a series of NPY analogues has been investigated in the present study. In particular, RP-HPLC techniques have been used as a probe for the putative amphipathic helical structure of NPY. The peptides comprised the parent molecule NPY[1–36], NPY[13–36], NPY[18–36] and a series of 16 analogues of NPY[18–36], specifically designed and synthesised as part of structure–function studies [32], which differed by single amino acid substitutions with the corresponding D-amino acid. Parameters which describe the hydrophobic contact area and the affinity of the NPY analogues for the non-polar ligands were determined over the temperature range of 4–80°C. The influence of ligand hydrophobicity was assessed by comparison of peptide retention with an *n*-butylsilica (C_4) and an *n*-octylsilica (C_8) sorbent. The results provide further insight into the contribution which peptide secondary structure makes to peptide retention behaviour in RP-HPLC and the dominant effect which hydrophobic forces have on the stabilisation of peptide structures in solution.

MATERIALS AND METHODS

Chemicals and reagents

Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile and 2-propanol (HPLC grade) were obtained from Mallinckrodt (Paris, KY, USA) and trifluoroacetic acid (TFA) from Auspep (Parkville, Australia). N-Acetyl-L-phenylalanine ethyl ester and angiotensin III were obtained from Sigma (St. Louis, MO, USA). Porcine NPY[18–36] analogues were synthesised by manual solid phase methods using *tert.*-butoxycarbonyl (Boc)-protected α-amino acids and included peptides with single D-amino acid substitutions at positions 18, 19, 20, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36. The analogues NPY and NPY[13–36] were obtained from Chiron Mimotopes (Clayton, Australia). All peptides were characterised by amino acid analysis and liquid secondary ion mass spectrometry as previously described [32].

Apparatus

Chromatographic measurements were made using a Waters Assoc. (Milford, MA, USA) liquid chromatograph consisting of two Model 501 solvent-delivery pumps, a U6K universal injector, a WISP Model 712 sample processor and an M680 automated gradient controller. Detection was carried out with an M440 detector coupled to an extended wavelength module and set at 215 nm, an ABB SE120 chart recorder and a HP-3396A integrator (Hewlett-Packard, Germany). Column temperatures were controlled using an ICI TC 1900 column oven (ICI Instruments, Dingley, Australia) or by immersion of the column in a thermostatted column water jacket coupled to a recirculating cooler (FTS Systems, NY, USA).

Reversed-phase chromatography was carried out with a Bakerbond butylsilica and octylsilica stationary phase (J.T. Baker, Phillipsburg, NJ, USA) with nominal particle diameters of 5 μm and average pore size of 30 nm packed into 250 × 4.6 mm I.D. cartridges.

Chromatographic procedures

Bulk solvents were filtered under vacuum and degassed by sparging with helium. Linear gradient elution was performed using 0.1% (v/v) TFA in deionised water (buffer A) and 0–100% of 0.09% (v/v) TFA with 50% (v/v) aqueous acetonitrile (buffer B) or 0–90% of 0.09% TFA in 40% (v/v) aqueous 2-propanol over gradient times of 30, 45, 60, 90, 120 min at a flow-rate of 1 ml/min. Peptide solutions were prepared by dissolving the solute at concentrations of 1 mg/ml in 0.1% TFA and stored until used at −20°C. Injection size varied between 10–50 μl (10–50 μg). The column dead time (t_0) was calculated as the retention time of the unretained solute

sodium nitrate and the gradient elapse time was determined from the difference between the column dead time and the onset of mobile phase breakthrough as monitored on the M440 detector as an absorbance increase. All experimental measurements were carried out in duplicate. Gradient retention data were analysed using the Pekenese program developed in this laboratory and written in Pascal for IBM compatible computers. The S and $\log k_0$ values was derived from the $\log \bar{k}$ *versus* $\bar{\psi}$ (see Results and Discussion section) plots using this established programme [3,33] and statistical analysis involved ANOVA linear regression analysis. The correlation coefficients for the linear regression were typically between 0.95–0.99.

Circular dichroism analysis

Circular dichroism (CD) measurements were carried out using an AVIV Model 62DS CD spectrophotometer (AVIV Assoc., Lakewood, NJ, USA) with scans between 185–250 nm. NPY[13–36] was dissolved in 0.02% TFA, 25 m*M* sodium dihydrogen phosphate, pH 7.0 at a concentration of 200 μg/ml.

RESULTS AND DISCUSSION

Dependence of retention on organic solvent concentration

Under conditions of linear gradient elution, the linear solvent strength model can be used to derive chromatographic parameters related to the physico-chemical properties of the solute molecule [3]. For conditions where regular reversed-phase gradient elution applies, a linear relationship between the median capacity factor, $\log \bar{k}$, and the median organic mole fraction, $\bar{\psi}$, is given by [34]

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

The values of S and $\log k_0$ can be determined by linear regression analysis of $\log \bar{k}$ *versus* $\bar{\psi}$ plots according to eqn 1. The significance of the S and $\log k_0$ values lies in their relationship, via the solvophobic theory [35,36], to the magnitude of the chromatographic contact area established by the peptide solute and the affinity which this peptide surface has for the chromatographic ligands. In order to study the effect of structural substitutions within NPY on its interaction with hydrophobic surfaces, gradient elution data were obtained for the series of peptides related to NPY listed in Table I. Gradient elution profiles were generated at gradient times between 30–120 min at a flow-rate of 1 ml/min and at temperatures of 4, 25, 37, 60 and 80°C with linear gradients from water containing 0.1% TFA to water–acetonitrile containing 0.09% TFA. The peptides were also chromatographed on C_4 and C_8 sorbents in order to study the influence of differences in ligand hydrophobicity. In addition, the role of the organic modifier was examined through analysis of data generated using 2-propanol as the organic modifier.

A total of 16 NPY[18–36] analogues differing by the substitution with the corresponding D-amino acid for the L-isomer at various sequence

TABLE I

PHYSICAL DATA FOR SOLUTES

Solute	Abbreviation	Sequence	Molecular mass
N-Acetylphenylalanine methyl ester	Ac-F	N-Ac-F-OMe	210
Angiotensin III	ANG-III	RVYIHPF	931
Neuropeptide Y[18–36]	NPY[18–36]	ARYYSALRHYINLITRQRY-NH_2	2090
Neuropeptide Y[13–36]	NPY[13–36]	PAEDLARYYSALRHYINLITRQRY-NH_2	2640
Neuropeptide Y	NPY	YPSKPDNPGEDAPAEDLARYYSALRH-YINLITRQRY-NH_2	3960

positions were examined. These L- to D- substitutions will affect the orientation of the amino acid side chains relative to the backbone of the peptide chain and the neighbouring amino acid side chains, but will not affect the amino acid composition. If a peptide solute interacts with the reversed-phase ligand without any specific orientation, D-substitutions would not be expected to exert any influence on the retention properties of these NPY analogues. However, it is well known that *cis* and *trans* prolyl peptide isomers are readily resolvable by RP-HPLC [37–39]. Moreover, it has also been known for many years that peptide diastereomers can be readily resolved by RP-HPLC [40]. Examination of Fig. 1a and b, which show plots of log $\bar{k}$ *versus* $\bar{\psi}$ for the series of NPY analogous separated on a C_8 sorbent with aqueous acetonitrile at 25°C, demonstrates that there were very specific orientation effects associated with the interaction of these peptides with the reversed-phase ligand. Similar results were also evident for the separation of these peptides on the C_4 sorbent and also for their separation with the C_8 sorbent and with 2-propanol as the organic modifier (results not shown).

For peptides containing up to approximately 15–20 amino acid residues with disorganised solution structures, the RP-HPLC retention times can be calculated with good precision according to the summated retention coefficients of the constituent amino acid residues [41,42]. However, when a peptide adopts a significant degree of secondary structure, the calculated retention time will deviate from the theoretical retention time based solely on the linear amino acid sequence. While there are no specific scales of amino acid retention coefficients available for the D-amino acids, it could be anticipated that all 16 NPY analogues should, in the absence of any orientation effects, have the same retention times under a given set of experimental conditions to those observed for the corresponding NPY[18–36] with all α-L-amino acids. From the experimental data in Fig. 1, it is apparent that there is significant resolution between a number of the NPY[18–36] analogues. Moreover, these results suggest that the interactive behaviour of these peptides is particularly sensitive to the

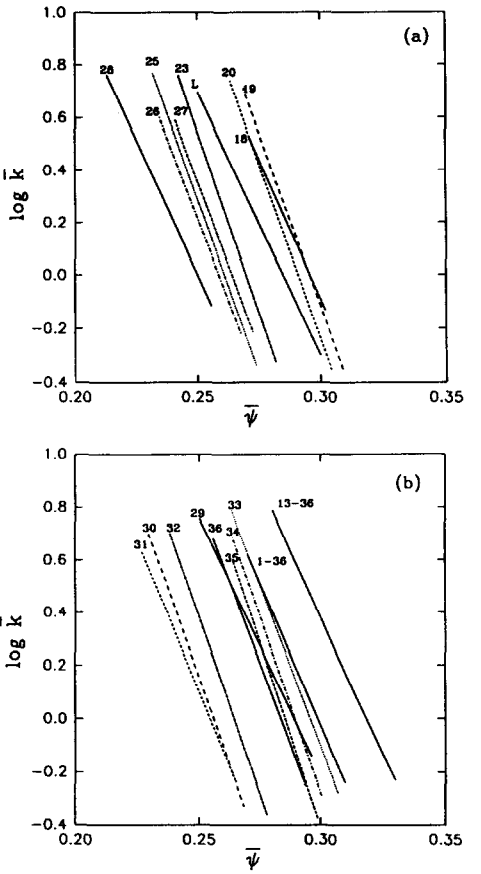

Fig. 1. Plots of log $\bar{k}$ *versus* $\bar{\psi}$ for NPY-related peptides separated on a C_8 sorbent with acetronitrile as the organic solvent modifier at 25°C. The plots were derived from best fit analysis to the data points (which have been excluded for clarity) ($r = 0.95–0.99$). Other chromatographic conditions are given in the Materials and Methods section. (a) Plots for the analogues with substitutions in the N-terminal region of NPY [18–36]; (b) plots for the analogues with substitutions in the C-terminal region. The retention plot for each D-substituted analogue is designated by the residue position of the D-amino acid substitution. L = Unsubstituted NPY[18–36]. The data are also shown for NPY[13–36] (13–36) and the native NPY[1–36] (1–36).

position of the D-amino acid substitution. Closer examination of the retention plots in Fig. 1 also reveals a trend in the elution behaviour of these peptide analogues relative to the retention plot for the all α-L-NPY[18–36]. The analogues with D-amino acid substitutions at the amino terminal region at positions Ala[18], Arg[19] and Tyr[20] and at the carboxy terminal region at positions Arg[33], Gln[34] and Arg[35] were all character-

ised by longer retention times than the corresponding all α-L-NPY[18–36]. D-Substitutions in the centrally located region at positions Ala[23], Arg[25], His[26], Tyr[27] and Leu[30], Ile[31] and Thr[32] in contrast exhibited much shorter retention times than the corresponding all α-L-NPY[18–36]. The amino acid sequence encompassing residues Glu[15]–Leu[30] of NPY is believed to exist in a helical conformation [21], whilst two-dimensional NMR and CD analysis have also shown that NPY[18–36] can adopt an α-helical conformation [30,31]. The amphipathic nature of this helix is clearly demonstrated in the helical Edmundson wheel shown in Fig. 2 for NPY[18–36] with the hydrophobic residues highlighted by the solid line. The observation (Fig. 1) that there is a significant shift in the log $\bar{k}$ *versus* $\bar{\psi}$ retention plots for the NPY[18–36] analogues when the D-amino acid substitution is displaced by 3–4 residues is consistent with this sequence adopting an amphipathic α-helical conformation capable of interacting with the stationary phase ligands through the hydrophobic face of the amphipathic helix (see Fig. 2). Substitution of the L-amino acid by the D-amino acid at sequence positions which are either located within the secondary structure or are involved in the stabilisation of this secondary structure will have resulted in the disruption of the amphipathic structure. The retention behaviour of the NPY analogues with D-substitutions at positions Ile[28] and Asn[29] clearly exhibited a deviation from this amphipathic pattern. Thus, the D-Ile[28] analogue exhibited the earliest retention of all 16 analogues, whilst the retention behaviour of the D-Asn[29] analogue was more representative of the later eluting (more hydrophobic) solutes but exhibited a different *S* value. Similar structural effects on peptide retention have also been observed by other workers [43,44] for synthetic peptides designed to adopt an amphipathic α-helical structure. Clearly, the D-substitutions have a significant effect on the interactive properties of these peptides through alteration of the characteristics of the chromatographic contact region. In order to probe the role of secondary structure in the retention behaviour of these peptides, the influence of temperature on the *S* and log k_0 values of these NPY analogues was consequently analysed.

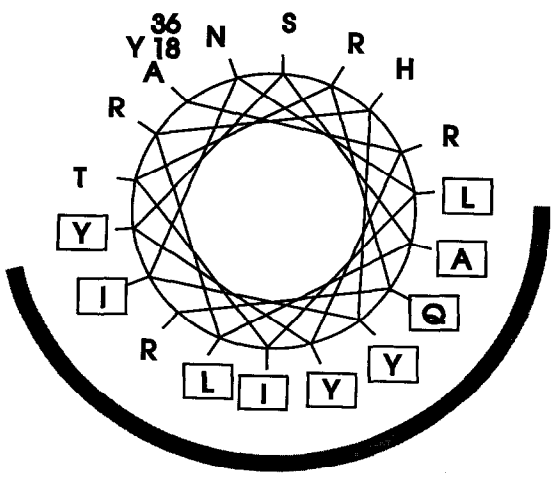

Fig. 2. Representation of the amino acid sequence of NPY[18–36] on an Edmondson helical wheel.

Dependence of S and log k_0 on temperature

C_8 Ligands/acetonitrile system

Retention behaviour of NPY, NPY[13–36] and NPY[18–36]. The value of the slope parameter, *S*, in RP-HPLC is related to the magnitude of the hydrophobic contact area established between the solute and the stationary phase ligands during the adsorption process [3,35,36]. Furthermore, the magnitude of the log k_0 value is a measure of the free energy changes associated with the binding of the solute to the stationary phase ligands in the absence of the organic modifier. Determination of these parameters for closely related peptides under a range of chromatographic conditions therefore provides the basis for the quantitative characterisation of differences in peptide orientation and affinity at the stationary phase surface. The conformation of peptides in solution is strongly influenced by temperature, a parameter which can be readily altered in chromatographic studies. The dependence of the *S* and log k_0 values on temperature for the polypeptides NPY, NPY[13–36] and NPY[18–36] are shown in Fig. 3a and b. The corresponding data for the control solutes N-acetylphenylalanine methyl ester (Ac-F) and angiotensin III (ANG-III) are also included for comparison. As Ac-F is a small molecule devoid

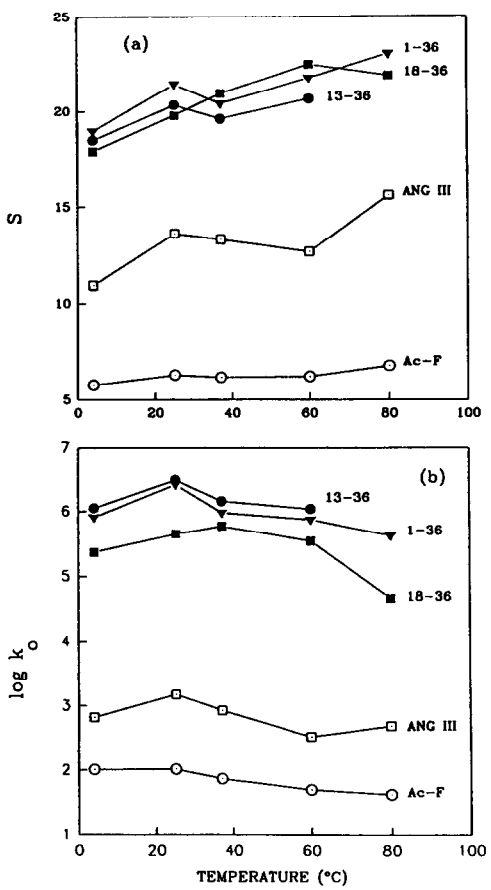

Fig. 3. Plots of (a) S and (b) log k_0 *versus* temperature for NPY, NPY[13–36], NPY[18–36], ANG-III, and Ac-F (see Table I) separated on a C_8 sorbent with acetonitrile as the organic solvent. See Materials and Methods section for other chromatographic details.

of secondary structure, changes in its retention behaviour over the temperature range studied will therefore not be a consequence conformational changes in the interactive structure. Similarly ANG-III is a small peptide that possesses minimal secondary structure but has been shown to be capable of adopting a partially-folded structure characterised by a *trans* His–Pro bond [45]. As evident from the data shown in Fig. 3, the S and log k_0 values for these control solutes were smaller in magnitude than the corresponding values obtained for the NPY related peptides. Moreover, there was very little change in the magnitude of the S and log k_0 values of these control solutes over the temperature range employed in these studies.

The experimental S values for all three NPY peptides were very similar which suggests that the magnitude of the chromatographic contact area is similar for these three peptides. The S value also increased with increasing temperature which is consistent with an increase in the size of the interactive region. The trends in the log k_0 values over the experimental temperature range showed a different relationship as can be seen in Fig. 3b. Firstly, there was an increase in the log k_0 values between 4 and 25°C which mirrors the increase in S values over the same temperature range. However, between 25 and 80°C there was a sharp decrease in the log k_0 values. At 80°C the elution profile of NPY[13–36], but not NPY[1–36] or NPY[18–36] was characterised by significant band broadening and multiple peak formation. These results are indicative of a series of temperature-dependent transitions with the formation of several conformational intermediates during the chromatographic process. It therefore appears that the truncation of the first 12 amino acid residues from the NPY sequence significantly changes the interactive behaviour of NPY[13–36] relative to the parent NPY molecule. With the exception of the data at 37°C, the log k_0 values for NPY[18–36] were significantly smaller than those obtained for the longer peptides. Overall, these results suggest that a thermal transition occurs in the conformation of these peptides near to 25°C and that this transition is associated with an increase in the chromatographic contact area but a decrease in the affinity of the peptides for the stationary phase ligands. NPY has been shown to exist in solution with highly stabilised secondary structure [22]. The CD spectrum of NPY[13–36] in 0.02% TFA and 25 mM NaH_2PO_4 at 25°C is shown in Fig. 4 and indicates a strong negative ellipticity at 220 nm corresponding to an α-helical content of *ca.* 35%. These results therefore suggest that the thermal transition observed at 25°C may arise from the disruption of the hydrophobic binding domain formed from an α-helical structure existing in solution or induced at the liquid–solid interface.

Retention behaviour of the D*-substituted analogues.* Fig. 5a and b show plots of the S and log k_0 values for each D-substituted NPY[18–36]

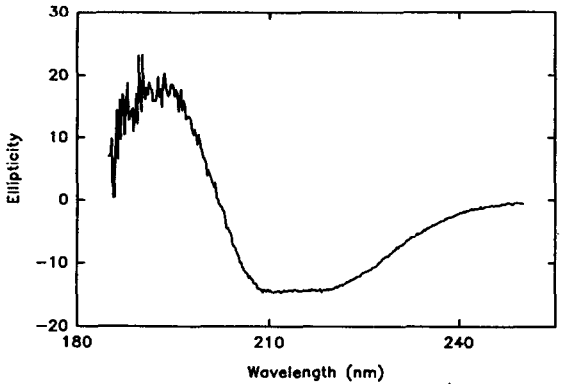

Fig. 4. Circular dichroism spectrum of NPY[13–36] measured in 0.02% TFA–25 m*M* Na_2PO_4. Analysis of the spectrum gave the following structural assignments: 35% α-helix, 23% β-turn, 26% random structure, 16% β-sheet.

analogue plotted against the residue position for the experiments carried out with the C_8 ligand and acetonitrile as the organic solvent modifier. At the lowest temperature examined, no significant variation in the S and log k_0 values was observed for all these NPY[18–36] analogues except for the D-Asn[29] analogue which showed an increase in both parameters. Thus the influence of the D-substitution on the relative contact area and affinity of these NPY[18–36] peptide analogues does not appear to be significant at 4°C. These results suggest that at this temperature, the peptides may exist in a relatively rigid conformation similar to that shown by the corresponding α-L-NPY[18–36].

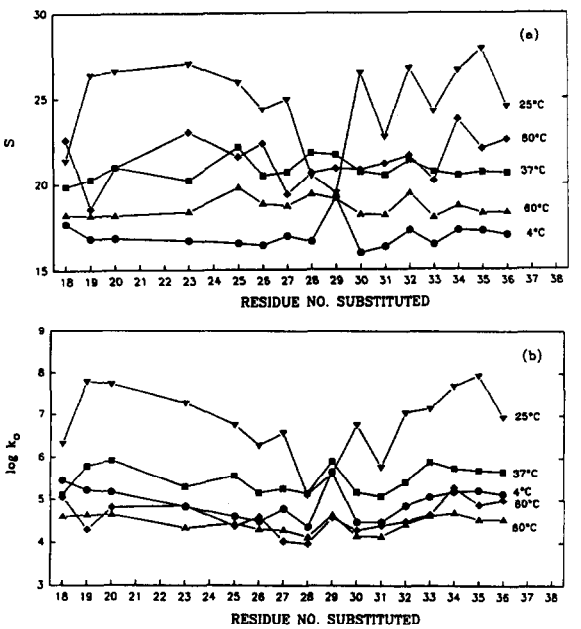

Fig. 5. Plots of (a) S and (b) log k_0 *versus* position of the D-amino acid substitution in NPY[18–36]. Peptides were separated on a C_8 sorbent and acetonitrile as the organic solvent modifier at the five temperatures shown. See Materials and Methods section for other chromatographic details.

A number of features relating to the conformation of the analogues are evident in the results obtained at 25°C. While the S and log k_0 values for D-Ala[18] was similar to the parent peptide, substitutions at amino acid residues from positions Arg[19] through to Tyr[27] resulted in a significant increase in both these parameters. Thus, the change in the chirality from L- to D- for the respective amino acid side chains has altered the characteristics of the chromatographic contact region and resulted in an increase in the contact area and the affinity. This trend for the D-isomers to have larger S and log k_0 values than the L-isomers was then disrupted by the dramatic decrease in these retention parameters when the D-substitution occurred at positions Ile[28] and Asn[29]. For example, the data obtained at 25°C indicate that the chromatographic contact region was severely disrupted when the D-substitutions are made in these sequence regions. This structural disruption continued to be evident as fluctuations in the retention parameters as the D-substitutions are made progressively throughout the C-terminal portion of the molecule.

When the measurements were carried out at 37°C, the chromatographic parameters were not significantly influenced by the D-amino acid substitutions as the majority of the NPY[18–36] analogues exhibited S and log k_0 values which were comparable to the corresponding α-L peptide. These results suggest that at this elevated temperature the unfolding of the secondary structure of these NPY[18–36] analogues which existed at 25°C was complete. Similar results were obtained when analogous experiments were carried out at 60 and 80°C with little fluctuation in the S values for all analogues. However, the log k_0 values for the NPY[18–36] analogues with D-substitutions at sequence positions between amino acid residues Asn[29]–Gln[34] showed variation at these elevated temperatures. In

summary, Ile[28] and Asn[29] appear to be important amino acid residues for the maintenance of the chromatographic contact region in the NPY[18–36] peptides since L- to D- substitution of these residues resulted in significant variation of the S values over the temperature range studied. At higher temperatures the magnitude of the S value of these NPY[18–36] analogues corresponded to the values for the other NPY analogues, suggesting that all peptides exist in a similar extended conformation at temperatures in excess of 25°C.

C_4 Ligands/acetonitrile system

Retention behaviour of NPY, NPY[13–36] and NPY[18–36]. The dependence of the S and log k_0 values on temperature for NPY, NPY[13–36] and NPY[18–36] and the control solutes separated on the C_4 sorbent with acetonitrile as the organic modifier are shown in Fig. 6a and b. These plots indicate that for NPY, there is an initial increase in both parameters between 4 and 25°C followed by a steady decrease in their value between 25 and 80°C. This trend contrasts to the behaviour observed for NPY[13–36]. Between 4 and 24°C, the S and log k_0 values for NPY[13–36] were similar to those observed for NPY[1–36]. Over this temperature range, NPY[18–36] and NPY[1–36] eluted from the C_4 sorbent as a single, nearly symmetrical peak. However, at temperatures above 25°C, the chromatographic profile of NPY[13–36] exhibited a second earlier eluting peak which increased in area relative to the original later eluting peak as the temperature was progressively increased. The plot shown in Fig. 6 for NPY[13–36] corresponds to the data for the original later eluting peak. These results are consistent with the involvement of a conformational transition similar to that observed when this peptide was eluted with the C_8/acetonitrile system. However, the appearance of only two well-resolved chromatographic peaks rather than the more complex multiplet observed with the C_8/acetonitrile system demonstrates that the conformational interconversion is strongly influenced by the nature of the immobilised ligand. Recent NMR relaxation experiments [46] and molecular dynamics studies [47] have revealed that the C_4 alkyl chain exists in a more

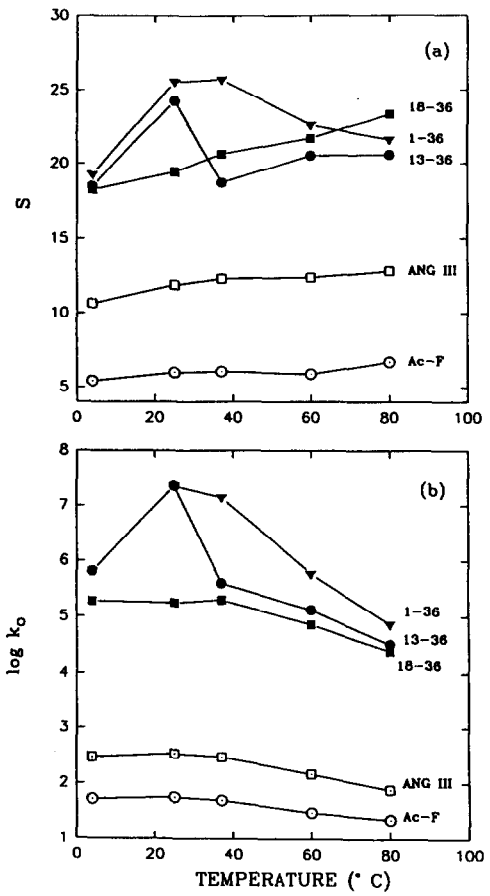

Fig. 6. Plots of (a) S and (b) log k_0 *versus* temperature for NPY, NPY[13–36], NPY[18–36], ANG-III and Ac-F separated on a C_4 sorbent with acetonitrile as the organic solvent. See Materials and Methods section for other chromatographic details.

rigid conformation than the C_8 and C_{18} ligands in *n*-alkylsilicas. These differences in ligand dynamics and hydrophobicity clearly have a significant effect on the interactive behaviour and relative conformational stability of the NPY peptides.

Retention behaviour of the D-substituted analogues. The dependence of the S and log k_0 values on temperature for the 16 NPY analogues with D-amino acid substitutions separated on a C_4 stationary phase with acetonitrile as the organic modifier are shown in Fig. 7a and b. At 4°C there was a small decrease in both parameters when D-substitutions were made between amino acid residues Ala[18] and His[26], compared to the values determined for the corre-

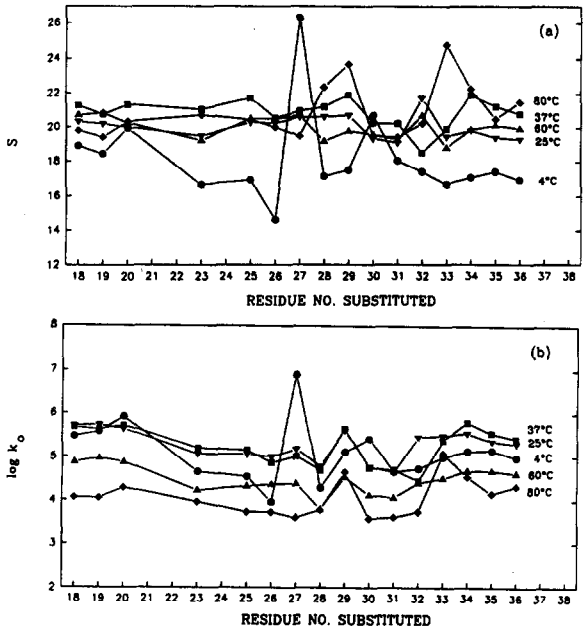

Fig. 7. Plots of (a) S and (b) log k_0 *versus* position of the D-amino acid substitution in NPY[18–36] analogues separated on a C_4 sorbent with acetonitrile as the organic solvent modifier at the five temperatures shown. See Materials and Methods section for other chromatographic details.

sponding α-L-NPY[18–36]. Much larger fluctuations in the magnitude of the S and log k_0 values were evident when D-substitutions were made between amino acid residues Tyr[27] and Ile[31]. This behaviour was then followed by essentially constant S and log k_0 values for the D-substitutions in the C-terminal positions.

At the higher temperatures of 25, 37 and 60°C, the S and log k_0 values were similar in magnitude to those observed at 4°C and there were only small fluctuations in these parameters for the various D-substituted NPY[18–36] analogues. At 80°C, with the exception of small increases in the S values for the analogues D-Ile[28], D-Asn[29] and D-Arg[33], the S values were similar to the values determined at 25°C, while the log k_0 values were consistently lower. It is apparent from these data that the substitutions by D-amino acids in the central region of the NPY[18–36] peptide cause significant changes in the interactive behaviour of these peptides with the C_4 ligand similar to those which were evident with the C_8 ligand. However, these changes in retention properties occurred at a lower temperature with the C_4 sorbent, which suggests that the longer *n*-octyl ligands may be able to more effectively stabilise the secondary structure of the peptides relative to the shorter more rigid *n*-butyl ligand. Furthermore, the most striking changes in the S and log k_0 values evident for the NPY[18–36] analogues eluted from the C_8 ligands corresponded to a large decrease in these parameters when the D-substitution occurred at amino acid residues Ile[28] and Asn[29]. In contrast, a large increase in these parameters was observed with the C_4 ligand for these two D-substituted analogues. These results indicate that the extent of the secondary structure adopted by these analogues is different with these two stationary phase ligands again demonstrating the important role of the ligand in the retention process.

C_8 Ligands/2-propanol

Retention behaviour of NPY[1–36], NPY[13–36] and NPY[18–36]. The interaction of peptides with chromatographic surfaces is dependent on the nature of both the immobilised ligand and the composition of the mobile phase. The effect of the organic modifier on the chromatographic behaviour of the NPY analogues was examined by carrying out similar gradient and temperature experiments with 2-propanol. 2-Propanol has a lower dielectric constant and solvent polarity, and hence greater eluotropic strength relative to acetonitrile (Table II). Therefore, the contribution of charge–charge, charge–dipole or dipole–dipole interactions between the peptide solute and the stationary phase with gradients of aqueous 2-propanol will be different to those occurring in aqueous acetonitrile. Solvent changes may also influence the conformation of peptides in solution which will

TABLE II

DIELECTRIC CONSTANT AND SOLVENT POLARITY OF MOBILE PHASE SOLVENTS

Solvent	Dielectric constant	Solvent polarity
2-Propanol	20.3	4.0
Acetonitrile	37.5	5.8
Water	80.0	10.2

in turn affect their interactive behaviour. In addition, the polarity of the mobile phase has been shown to influence the mobility of the stationary phase ligands which will also influence the retention behaviour of peptides. In the present series of experiments data could not be collected at 4°C due to the excessive pressure drop associated with the use of 2-propanol at this temperature. Fig. 8a and b show plots of the dependence of S and log k_0 values for the NPY related peptides NPY[1–36], NPY[13–36] and NPY[18–36] with the C_8 sorbent and 2-propanol as the organic modifier between 15–80°C. As evident from these results, an increase in the S values was accompanied by a decrease in the log k_0 values over the temperature range studied.

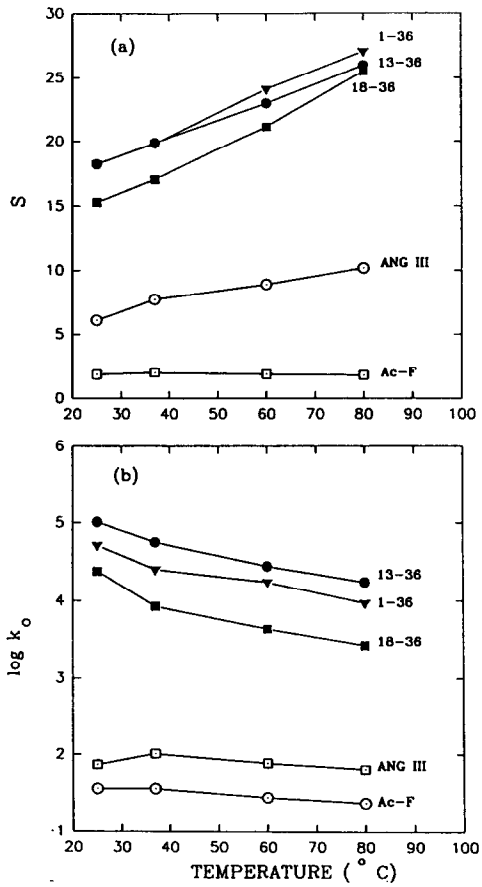

Fig. 8. Plots of (a) S and (b) log k_0 *versus* temperature for NPY, NPY[13–36], NPY[18–36], ANG-III and Ac-F separated on a C_8 sorbent with 2-propanol as the organic solvent. See Materials and Methods section for other chromatographic details.

These results are similar to the data derived for the C_8/acetonitrile system except that lower log k_0 values were observed for the C_8/2-propanol system. As the change in these parameters of the NPY peptides was larger than the changes observed for the control solutes, it appears that the increases in the interactive contact area and the corresponding decrease in the affinity term are associated with a significant change in the conformation of these peptides over the experimental temperature range. 1-Propanol has been shown by other workers [48] to induce surface-associated conformational changes of proteins bound to a C_8 alkyl silica sorbent, and similar effects may also be induced in the presence of 2-propanol.

Retention behaviour of the D*-substituted analogues.* The dependence of the S and log k_0 values on temperature for the 16 NPY[18–36] analogues is illustrated in Fig. 9a and b. Generally, an increase in the S value occurred as the temperature was increased from 25 to 80°C, while there was little change in the magnitude of the log k_0 values over this temperature range. In

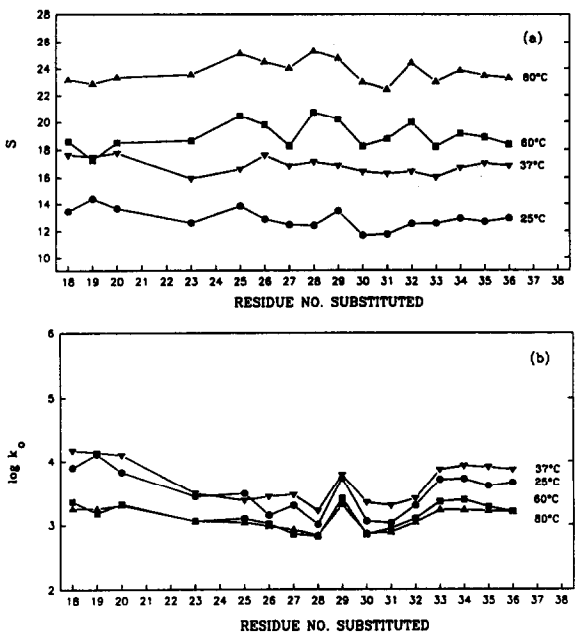

Fig. 9. Plots of (a) S and (b) log k_0 *versus* position of the D-amino acid substitution in NPY[18–36] analogues separated on a C_8 sorbent with 2-propanol as the organic solvents at the four temperatures shown. See Materials and Methods section for other chromatographic details.

addition, at both 25 and 37°C, the S and log k_0 values were very similar for all D-substituted analogous. However, small fluctuations in the S value for the NPY[18–36] analogues with D-substitutions in the central region between amino acid residues Tyr[27]–Thr[32] were apparent at the higher temperatures. Overall, these results are significantly different to the results observed for the C_8/acetonitrile and the C_4/acetonitrile system. It is possible that 2-propanol may stabilise the conformation of the peptides relative to acetonitrile resulting in smaller deviations in the experimentally observed S and log k_0 values. Conversely, it is also possible that the peptides adopt a less well-defined secondary structure in the presence of 2-propanol compared to the structure in acetonitrile and the increases in the contact area term simply correspond to an overall expansion of the molecular conformation. Analysis by circular dichroism of the conformation of these peptides in these solvent systems would help to distinguish between these alternatives. Results of such experiments will be reported subsequently.

CONCLUSIONS

In the present study, the exquisite sensitivity of RP-HPLC to resolve peptide structural isomers has been clearly demonstrated. The ability to discriminate between analogues of NPY[18–36] differing solely by the chiral inversion of a single residue indicates that the stationary phase ligands act as a probe of peptide surface topography. In addition, it is clear from the presented data that the interaction of peptides with the RP-HPLC sorbent is strongly dependent on the spatial arrangement of the amino acid residues rather than simply the overall amino acid composition. It has been suggested that the ability of a peptide hormone to form an amphipathic α-helix and bind to phospholipids may be related to the biological potency of the peptide [49]. The ability to study the interactive behaviour of peptides at hydrophobic surfaces represents a powerful approach to characterising the molecular features which control the orientation and binding of peptides with different surfaces. Chromatography is a dynamic method which can be used for the study of the adsorption of peptides at solid–liquid interfaces. In the present investigations, these attributes have been exploited to permit the rapid RP-HPLC analysis of the surface structure of NPY-related peptides. These results supplement data derived from spectroscopic analyses and biological activity measurements, permitting collectively a more detailed understanding of structure-function relationships of these regulatory peptides to be established.

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