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Evaluation of peptide–peptide interactions using reversed-phase high-performance liquid chromatography

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ABSTRACT

The separation of peptides during RP-HPLC depends mainly upon differential hydrophobic interactions of the individual peptides being separated with the C_{18} group of the stationary phase. We have examined the behavior of dimeric disulfide-linked model peptides during RP-HPLC in order to study self-induced conformational effects. A set of 18 analogues of the amphipathic α-helical sequence Ac-LKLLKKLLKKLKKLLKKL-NH_2 was used for this study. These analogues differed only by the successive replacement of each position with a cysteine. Strong peptide–peptide interactions, occurring through interchain hydrophobic forces, resulted in a presenting face to the C_{18} group, consisting primarily of lysine residues and, in turn, in early retention times. Three homo-dimers were also found to be strongly α-helical in water as determined by circular dichroism spectroscopy.

INTRODUCTION

The induced secondary structures of peptides, such as amphipathic α-helicity [1], are known to be essential for many peptide–peptide or peptide–protein interactions. Examples include the binding of melittin [2], or specific model amphipathic peptides [3,4], to calmodulin. Amphipathicity, as well as other general structural effects such as dipole–dipole interactions [5], hydrophobic interactions [6], and salt bridge formation [7], have also been established as important stabilizing elements in peptide and protein tertiary and quaternary structures.

Hydrophobic forces, which dominate peptide–peptide interactions as well as protein folding and stability, are generally counterbalanced by the solvation of hydrophilic residues which renders the proteins soluble in water. As a result, in globular proteins hydrophilic external surfaces interact with and are solvated by water, whereas hydrophobic amino acids are "buried" and form a lipid-core environment [6]. Often, hydrophilic–hydrophobic amino acid segregation follows an initial non-specific binding of a peptide to a protein, lipid, cell membrane, etc. This type of induced segregation of hydrophilic and hydrophobic amino acids is suggested for a variety of important physiological processes, such as hormone–receptor [8] and T-cell–major histocompatibility complex (MHC) interactions [9]. For instance, using disulfide-linked peptide dimers, O'Shea *et al.* [10] was able to mimic the conformations of proximal sequences (not covalently linked) found in native proteins. Others have designed and synthesized artificial "proteins" whose overall structures are formed and/or stabilized by hydrophobic interactions [11–13]. Recently, Hahn *et al.* [14] reported the *de novo* chemical synthesis of a 4-barrel helix bundle having enzymatic activity.

In earlier studies carried out in this laboratory, induced conformational factors (especially amphipathic α-helical arrangements) were found to influ-

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ence the retention behavior of peptides during reversed-phase high-performance liquid chromatography (RP-HPLC) [15–18]. This earlier work has lead us to the general hypothesis that every peptide has a small number of energetically favored conformations which are induced by the interaction of the peptide with the hydrophobic groups of the stationary phase. For instance, three peptides having the same composition (nine leucine and nine lysine residues) but different linear sequences representing several potential structural motifs, eluted over a range of 20 min [17]. Those which adopted classically and segmentally amphipathic α-helical conformations eluted later than anticipated [19]. By systematically studying the RP-HPLC behavior of single substitution analogues of a given sequence, we were able to evaluate the induced conformation of this peptide [17,18,20]. In particular, the model peptide Ac-LKLLKKLLKKLKKLLKKL-NH_2 was found to be induced into a classically amphipathic α-helical conformation during RP-HPLC [17,20]. Since an important factor in peptide–peptide and peptide-protein interactions involves conformational effects induced by hydrophobic constituents, we have investigated in the present study the use of RP-HPLC for the study of such interactions. To facilitate the study of such peptide–peptide interactions, we have synthesized a complete series of individual cysteine substitution analogs of the model peptide studied in earlier studies: Ac-LKLLKKLL-KKLKKLLKKL-NH_2. We then examined peptide–peptide interactions of these dimeric disulfide-linked model peptides using RP-HPLC, along with circular dichroism spectroscopy (CD) as a complementary and contrasting means of investigation. Since this set of peptide analogues represents two closely related compositions (*i.e.*, either 9 leucines, 8 lysines and 1 cysteine or 8 leucines, 9 lysines and 1 cysteine), only two distinct retention times would be expected to be seen if conformation was not a factor contributing to elution behavior [19]. Our working hypothesis, however, was that dimerization of each individual monomer by disulfide bridge formation would result in homo-dimeric peptides, which through self interactions would be induced into specific secondary structures. The overall surface hydrophobicity of these cysteine homo-dimers would be expected to differ depending on the conformation of the two peptide chains, which in turn would be dependent on the position of the cysteine.

MATERIALS AND METHODS

Peptide synthesis

Peptides were prepared by simultaneous multiple peptide synthesis (SMPS) [21]. Final cleavage and deprotection were carried out with liquid hydrogen fluoride (HF), using Tam *et al.* "low–high" HF cleavage protocol [22] with a 24-vessel cleavage apparatus [23] (Multiple Peptide Systems, San Diego, CA, USA). Oxidized peptides were produced by stirring a solution of the peptides (5 mg/ml) in 0.1 *M* NH_4HCO_3, pH 8, overnight at room temperature. The peptides were purified prior to CD studies using a DeltaPrep 3000 preparative RP-HPLC with a Foxy Fraction collector (Millipore, Waters Division, San Francisco, CA, USA). Analytical RP-HPLC was used to determine which fractions of the desired purity were to be pooled and lyophilized. The identities of the peptides were confirmed by time-of-flight mass spectroscopy analyses on a BIOION 20 spectrometer.

Analytical RP-HPLC

Relative retention times were determined using a Beckman gradient HPLC system consisting of two Model 110A pumps, a Beckman Model 421 microprocessor (Beckman Instruments, Anaheim, CA, USA), a Hitachi Model 100-20 variable wavelength spectrophotometer (Baxter Scientific Products, Los Angeles, CA, USA), a Shimadzu C-R3A Integrator (Cole Scientific, Calabasas, CA, USA), and a Bio-Rad Model AS-48 autosampler (Bio-Rad Laboratories, Richmond, CA, USA). Samples (20 μl, 0.2 mg/ml) were analyzed on Vydac 218TP54 C_{18} columns (Alltech Associates, Los Altos, CA, USA) (250 mm × 4.6 mm I.D., 5μm). Peptide elution was monitored at 215 nm. Buffer A consisted of 0.05% trifluoroacetic acid (TFA) in water and buffer B consisted of 0.05% TFA in acetonitrile. The peptides were analyzed using a 1%/min increasing gradient, starting at 5% buffer B.

RP-HPLC determinations of the monomeric forms were carried out by adding 50 μl of 5 m*M* dithiothreitol to a RP-HPLC sample of peptide (500 μl of a 1 mg/ml solution) in 0.1 *M* NH_4HCO_3, pH 8 [24]. After 1 h at room temperature, the pH was lowered prior to RP-HPLC by the addition of 50 μl of 10% AcOH. The samples were then analyzed by analytical RP-HPLC.

Circular dichroism measurements

All measurements were performed at ambient temperature on a Jasco J-720 circular dichroism spectrometer (Jasco, Easton, MD, USA). The instrument was routinely calibrated with an aqueous solution of ammonium [$^2H_{10}$]camphorsulfonic acid. Constant nitrogen flushing was employed. The measurements were carried out using quartz cells of 0.1 cm pathlength at a peptide concentration of 0.050 mg/ml (*i.e.*, $1.2 \cdot 10^{-5}$ mol/l). The relative concentration of each peptide was determined by its UV absorption at 210 nm prior to the measurements. The mean residue ellipticities ([θ]) were calculated using the relationship $[\theta] = 100/cnl$, where θ is the ellipticity (mdeg), c is the peptide concentration (m*M*), *n* is the number of residues in the peptide, and *l* is the pathlength (cm). The approximate percent helicity for the homo-dimeric peptides was calculated using $[\theta]_{222}$ (at 222 nm) with the assumption that a 100% helical peptide yields $[\theta]_{222} = -33\,400$ deg cm^2 dmol^{-1} [25].

TABLE I

PEPTIDE SEQUENCES

Sequence	Substituted residue
Ac-LKLLKKLLKKLKKLLKKL-NH_2	None
Ac-CKLLKKLLKKLKKLLKKL-NH_2	L-1
Ac-LCLLKKLLKKLKKLLKKL-NH_2	K-2
Ac-LKCLKKLLKKLKKLLKKL-NH_2	L-3
Ac-LKLCKKLLKKLKKLLKKL-NH_2	L-4
Ac-LKLLCKLLKKLKKLLKKL-NH_2	K-5
Ac-LKLLKCLLKKLKKLLKKL-NH_2	K-6
Ac-LKLLKKCLKKLKKLLKKL-NH_2	L-7
Ac-LKLLKKLCKKLKKLLKKL-NH_2	L-8
Ac-LKLLKKLLCKLKKLLKKL-NH_2	K-9
Ac-LKLLKKLLKCLKKLLKKL-NH_2	K-10
Ac-LKLLKKLLKKCKKLLKKL-NH_2	L-11
Ac-LKLLKKLLKKLCKLLKKL-NH_2	K-12
Ac-LKLLKKLLKKLKCLLKKL-NH_2	K-13
Ac-LKLLKKLLKKLKKCLKKL-NH_2	L-14
Ac-LKLLKKLLKKLKKLCKKL-NH_2	L-15
Ac-LKLLKKLLKKLKKLLCKL-NH_2	K-16
Ac-LKLLKKLLKKLKKLLKCL-NH_2	K-17
Ac-LKLLKKLLKKLKKLLKKC-NH_2	L-18

RESULTS

Peptide Synthesis

A set of 18 analogues of the peptide Ac-LKLLKKLLKKLKKLLKKL-NH_2, found in earlier studies to be induced into a classically amphipathic α-helical conformation during RP-HPLC [17,20], was synthesized. This starting sequence is illustrated in helical wheel [26] and lateral representations in Fig. 1. The analogues differed only in the successive replacement of each position with a cysteine (Table I). Each peptide was prepared by the SMPS method [21]. The homo-dimers contained in the crude synthetic peptides ranged from 20 to 80% of the total.

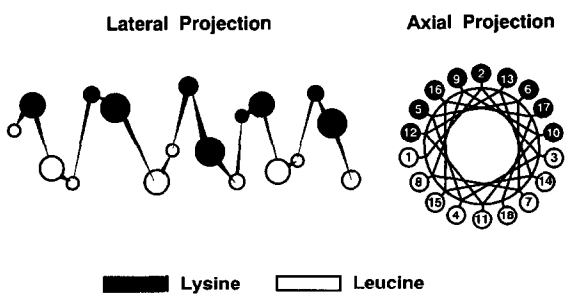

Fig. 1. Helical wheel and lateral representation of Ac-LKLLKKLLKKLKKLLKKL-NH_2

Peptide–peptide interactions as estimated by RP-HPLC

The homo-dimers contained in the crude synthetic peptides were readily reduced with dithiothreitol [24], enabling the determination of the respective retention times of both the monomeric and homo-dimeric forms of each analogue. The relative retention times for these analogues were the same in three separate determinations. The retention times of the monomeric and homo-dimeric forms (Table II) were plotted from the N-terminus relative to the position of the cysteine residue in the peptide (Fig. 2). A larger, overall variation in retention times (35 min) was observed for the homo-dimers than for the monomers (10 min). When the cysteine was located on the lysine side of this amphipathic α-helix, except for the extreme N- and C- terminal residues, both the monomeric and homo-dimeric forms eluted later than the starting peptide (X in Fig. 2). In contrast, when the cysteine was located on the leucine side of the amphipathic α-helix, all of the pep-

TABLE II

RETENTION TIME OF MONOMERS AND HOMO-DIMERS

The retention times were determined using a 1% gradient starting at 5% B.

Substituted residue	Retention time (min)	
	Monomers	Homo-dimers
None	46.2	
L-1	42.9	48.2
K-2	47.7	51.2
L-3	42.6	49.4
L-4	41.6	36.3
K-5	48.6	54.8
K-6	48.0	48.7
L-7	42.4	31.9
L-8	42.8	40.0
K-9	47.3	46.4
K-10	51.2	50.6
L-11	41.5	30.5
K-12	50.2	53.1
K-13	47.7	54.2
L-14	41.9	43.6
L-15	41.9	39.3
K-16	47.3	64.2
K-17	49.3	44.6
L-18	43.1	47.9

tides eluted earlier than the starting peptide. In particular, a substantially prolonged retention time for the homo-dimeric form was observed when lysine-16 was replaced with a cysteine, while much earlier retention times were found upon replacement of either leucine-7 or leucine-11.

If one envisions the hydrophobic alkyl groups of the amphipathic α-helix as being imbedded in the C_{18} layer of the stationary phase, then a more informative way to plot the results obtained relates to the position of the cysteine substitution in a helical wheel format. Thus, these substitutions were viewed counter-clockwise for those analogues in which a leucine has been replaced by a cysteine, and clockwise for those analogues in which a lysine was replaced by a cysteine (Fig. 3). In each case, the plot was started at the "nine o'clock" position (see helical wheel representation, Fig. 1). This representation emphasizes the effects of the position being replaced by the single cysteine residue relative to the location of the C_{18} interface. Large variations in retention times were observed in the 9 different homo-dimeric analogues in each set: the retention times varied by 20 min when either leucine or lysine was replaced by a cysteine (Fig. 3A). The retention times of those homo-dimers resulting from replacements of a leucine by a cysteine follow a pattern (except for position 18), which indicates that the closer a cysteine substitution is to the C_{18} interface, the longer its retention time (*i.e.*, the stronger its interaction with the C_{18}). In contrast, only small variations in retention times were found for the cor-

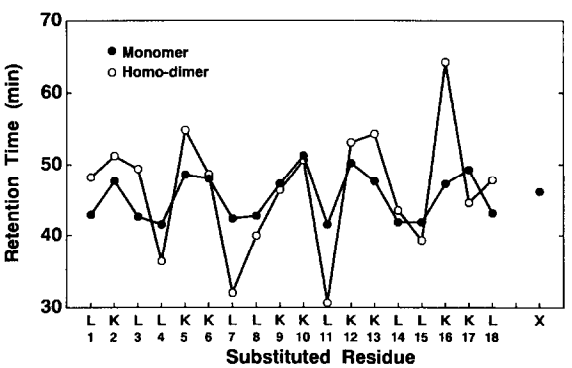

Fig. 2. RP-HPLC retention times of the monomeric and dimeric forms of the cysteine substitution analogues. The retention times were determined using a 1% gradient (5–75% B in 70 min). X = parent sequence.

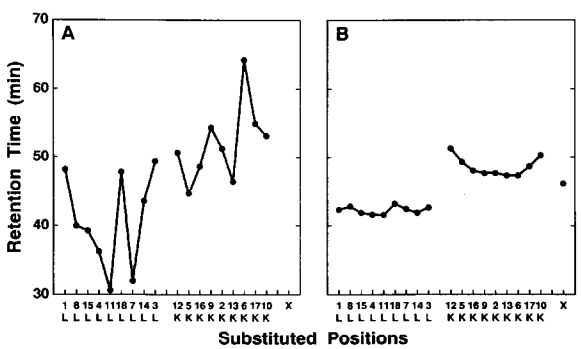

Fig. 3. Variation of the RP-HPLC retention times of the cysteine substitution analogues as viewed in a helical wheel representation. The substituted leucine positions are plotted as viewed counter-clockwise, while the substituted lysine positions are plotted as viewed clockwise on the helical wheel representation shown in Fig. 1. Both plots started at the "nine o'clock" position. (A) homo-dimeric forms; (B) monomeric forms; X = Parent sequence.

responding monomers (a 2-min and 3-min range when a cysteine replaced either a leucine or a lysine, respectively; Fig. 3B).

Peptide–peptide interactions as estimated by circular dichroism

CD spectroscopy has long been used for the estimation of the secondary structures of peptides or proteins in solution [27]. CD investigations were carried out to confirm or contrast the induced conformation predicted by RP-HPLC. Following air oxidation to their homo-dimeric forms in 0.1 *M* NH_4HCO_3, the analogues were purified to greater than 95% by RP-HPLC. RP-HPLC was used to confirm the absence of the monomeric forms of the peptides. Their dimeric states were confirmed by mass spectroscopy ($M+H=4443\pm2$ when a cysteine replaced a leucine, $M+H=4413\pm2$ when a cysteine replaced a lysine). The induced conformations of these homo-dimers were examined in salt-free aqueous solution. The CD spectra showed that only those analogues in which leucine-7, leucine-8, leucine-11, lysine-12 or leucine-18 were replaced with a cysteine adopted any significant percentage of defined secondary structure in aqueous solution (*i.e.*, α-helical conformation; Fig. 4 and Table III).

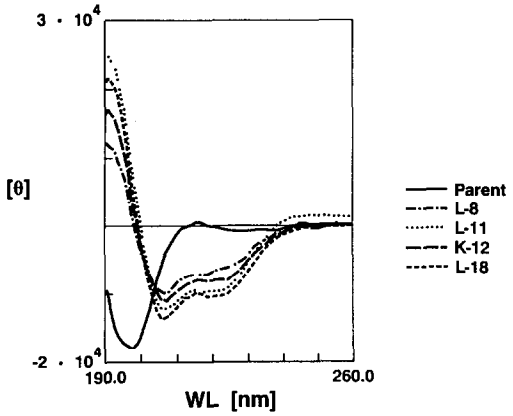

Fig. 4. Circular dichroism spectra in aqueous solution. The CD spectra show the mean residue molar ellipticities in deg cm^2 $dmol^{-1}$ as a function of wavelength (WL) for 0.050 mg/ml peptide in water.

TABLE III

α-HELICITY OF PEPTIDES AS ESTIMATED BY CD AT 222 nm

ND = Not determined. All of the peptides are in their dimeric form.

Substituted residue	% α-Helicity				
	H_2O	10% TFE[a]	20% TFE	30% TFE	40% TFE
None	>5	11	33	39	49
L-1	9	43	41	42	44
K-2	<5	37	42	47	58
L-3	<5	33	39	41	44
L-4	8	54	50	52	55
K-5	<5	53	54	ND	ND
K-6	<5	20	39	42	45
L-7	32	44	48	50	48
L-8	19	32	32	38	34
K-9	<5	18	29	30	40
K-10	11	31	49	43	46
L-11	29	46	54	60	58
K-12	24	34	35	38	ND
K-13	8	31	ND	42	42
L-14	<5	28	34	40	36
L-15	<5	37	41	44	50
K-16	<5	24	26	28	36
K-17	<5	21	22	ND	33
L-18	31	43	ND	45	43

[a] A v/v mixture of TFE in water.

TABLE IV

RELATIVE α-HELICITY AS DETERMINED BY CD USING RATIOS OF CD INTENSITIES [29]

All of the peptides are in their dimeric forms.

Substituted Residue	$R1 = [\theta]_{max}/[\theta]_{min}$		$R2 = [\theta]_{222}/[\theta]_{min}$	
	H_2O	10% TFE[a]	H_2O	10% TFE
None	0.52	−0.74	0.02	0.37
K-6	0.43	−0.85	0.10	0.50
L-7	−1.62	−2.16	0.79	0.88
K-9	0.46	−0.93	0.01	0.53
L-11	−2.00	−1.73	0.79	0.81
K-16	0.12	−1.92	0.15	0.73
K-17	0.54	−1.61	0.02	0.64
L-18	−1.58	−2.26	0.76	0.79

[a] A v/v mixture of TFE in water.

The propensity of the homo-dimeric analogues to adopt an α-helical conformation in the presence of trifluoroethanol (TFE), a solvent that induces helicity in potentially α-helical polypeptides [28], was then examined. The relative percentages of helicity for each analogue in the presence of increasing amounts of TFE are shown in Table III. The maximum helicity (ranging from 33% to 60%) was reached for each analogue in the highest percent TFE used (40%). The greatest amount of α-helicity was observed upon substitution of leucine-11 with a cysteine, while the lowest amount of α-helicity was found for those analogues in which a cysteine replaced a lysine at positions 16 or 17. Substitutions analogues at position 6 and 9, as well as the starting sequence, also had low helicity in 10% TFE.

To avoid misinterpretation of the data due to small variations in concentration, Bruch *et al.* [29] proposed the use of two parameters, $R1 = [\theta]_{max}/[\theta]_{min}$ and $R2 = [\theta]_{222}/[\theta]_{min}$. These ratios are based on the fact that in a two-state equilibrium between α-helix and random coil, the absolute value of $[\theta]_{222}$ and the maximum ellipticity $[\theta]_{max}$, increase relative to the minimum ellipticity $[\theta]_{min}$ as a function of α-helicity, independent of the peptide concentration. $R1$ values should decrease and $R2$ values should increase with an increase in α-helicity. For each peptide analogue studied, the CD spectra exhibited an isodichroic point at 203 nm as a function of %TFE, indicating a two-state equilibrium between a random coil and an α-helical conformation. The relative ratios calculated for the homo-dimer analogues confirmed that the highest percent α-helicity in water is reached when a cysteine replaced leucine-11, followed next by a cysteine replacement of leucine-7 and leucine-18 (lowest $R1$ and highest $R2$ values, Table IV). In 10% TFE, the low levels of helicity observed for the parent peptide and for those analogues in which a cysteine replaced either lysine-6 or lysine-9 were supported by high $R1$ and low $R2$ ratios (Table IV).

DISCUSSION

In anisotropic environments such as RP-HPLC, dimerization of the parent sequence by disulfide bridge formation results in homo-dimeric peptides that are induced into specific secondary structures. These specific self-induced conformations were entirely dependent on the location of the cysteine in the linear chain of the peptide, and in turn on the position of the peptide chains relative to one another. Since two closely related compositions are being studied (*i.e.*, either 9 leucines, 8 lysines and 1 cysteine or 8 leucines, 9 lysines and 1 cysteine), only two distinct retention times would be expected to be seen if conformation was not a factor contributing to elution behavior [19]. However, large variations (20 min) were observed in the retention times of the homo-dimers. This indicates that the specific secondary structures self-induced by peptide-peptide interactions are affecting RP-HPLC behavior. In support of this hypothesis, only small variations in retention times were found for the corresponding monomers (a 2-min and 3-min range when a cysteine replaced leucine or lysine, respectively; Fig. 3B).

The shortest retention time found for a homo-dimer resulted from replacing leucine-11 with a cysteine. This can be explained by the occurrence of strong peptide–peptide interactions through the two leucine faces of the two induced amphipathic helices. Since leucine-11 is located in the central region of the hydrophobic side of the amphipathic helix (see helical wheel representation, Fig. 1), this self-induced conformation results in a C_{18} surface presentation consisting almost entirely of lysine residues, and thus in an early retention time. The

adoption of an α-helical conformation for this analogue observed by CD in aqueous solution strengthens our belief that strong interchain hydrophobic interactions occur between the leucine residues, and in turn result in a stabilized double-stranded α-helical conformation. Similar but smaller effects are seen upon replacing leucine-7 with a cysteine.

The α-helical conformation calculated by CD in water for the substitution analogue of leucine-18 may result from the location of the cysteine at the far end of the chain. Thus, one chain may be folding over onto the other, again resulting in stabilization of a double-stranded α-helical structure through hydrophobic interactions between the leucine residues, as seen by Hodges and co-workers [11,12] for double-stranded coiled-coils conformation. However, the prolonged retention time found for this analogue relative to the others in this series indicates that an extended, or partially extended, conformation exists upon interactions between the leucines and the C_{18} group of the stationary phase.

In contrast to a folding process, the prolonged retention time observed when lysine-16 was replaced by a cysteine can be considered to be due to a juxtaposition of the two peptide chains in an antiparallel manner, resulting in a functionally extended C_{18} group hydrophobic presenting face (*i.e.*, equivalent to a 36-residue chain presenting 18 leucines on one face). This would result in a much stronger interaction of this dimer with the C_{18} group of the stationary phase. The low helical content found by CD for this analogue reconciles better with a single-stranded α-helical conformation than a double-stranded structure. The disulfide bridge appears, therefore, not to be contributing directly to its α-helical stability in aqueous solution.

In conclusion, we believe that RP-HPLC is a useful tool for the comparative study of self-induced conformations resulting from peptide–peptide interactions. The significant variations found between retention times of the homo-dimeric analogues studied here confirm the predominant role of amphipathicity in peptide–peptide interactions.

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