Amino Acids (1996) 10: 295–304

Amino
Acids
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Printed in Austria

Differential self assembly of amphiphilic helical peptides

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Accepted February 10, 1996

Summary. A series of amphiphilic, helical peptides was designed and synthesized to investigate the components necessary for formation of helical bundles with differing aggregation states. Minimalistic sequences were employed for the peptides which contained either four (**Leu4**), six (**Leu6**) or eight (**Leu8**) leucine residues within a sixteen amino acid sequence. All peptides were highly helical as evaluated by circular dichroism, and the helical content of each peptide exhibited a concentration dependence. Size exclusion chromatography confirmed aggregation states of dimer/trimer for **Leu4**, tetramer for **Leu6**, and hexamer octamer for **Leu8**. Disulfide crosslinking studies also confirmed that the dimer of **Leu4** favored a parallel orientation with respect to the helical dipole. This systematic study clearly defines the role of hydrophobicity in the self assembly of helical peptides; peptides with a small hydrophobic face favor small bundle sizes, whereas peptides containing larger hydrophobic faces form correspondingly larger helical bundles.

Keywords: Amino acids – Self assembly – Peptide – Amphiphilic – Helical

Introduction

Inherent in the use of peptide self assembly for *de novo* design of proteins with novel functions is a knowledge of the factors responsible for the formation of one unique assembly over another. To date researchers have designed helical bundles which contain the full array of differently sized aggregate structures. Initial research focused on the *de novo* design of four helix bundles (Eisenberg et al., 1986; Ho and DeGrado 1987; Hecht et al., 1990; Osterhout et al., 1992) and dimeric coiled coils (Lau et al., 1984; Zhou et al., 1992; Monera et al., 1993, 1994). More recently, Kim and coworkers have investigated the role of interfacial residues of coiled coil peptides in governing self assembly of dimeric, trimeric and tetrameric helical bundles (Harbury et al., 1994). The formation of larger helical bundles via templated structures (Sasaki and Kaiser, 1989; Lieberman and Sasaki, 1991; Akerfeldt et al., 1992; Mutter et al., 1992; Ghadiri et al., 1992) or by self assembly (Chin et al., 1992)

has also been reported. Due to the different lengths and amino acid compositions of the reported, self-assembling peptides, a unifying picture of the factors responsible for differential helical bundle formation is lacking. We report a systematic study on a series of highly similar peptides to address the role of exposed hydrophobic surface area in helix bundle formation in aqueous solution.

Materials and methods

Peptide synthesis

The peptides were synthesized using solid phase peptide synthesis with a fluorenylmethyloxycarbonyl (Fmoc)- based strategy (Chang and Meienhofer, 1978) on the Rink resin (Rink, 1987) in a stepwise manner using the 1-hydroxybenzotriazole (HOBt) method (Mojsov and Merrifield, 1981). The Fmoc-amino acids were purchased from Bachem Biosciences, the Rink resin from Nova Biochem, HOBT from Lancaster Synthesis, and all remaining chemicals were purchased from Aldrich.

Each amino acid was incorporated using two 1 hr couplings with a 0.3 M solution of amino acid and HOBT in N-methylpyrrolidinone, and an equimolar amount of diisopropylcarbodiimide (0.3 M) in CH_2Cl_2. After the coupling reaction, the resin was treated with a 0.3 M solution of acetic anhydride and diisopropylethylamine in CH_2Cl_2 to block any unreacted amino groups. Prior to the next coupling step, the N-terminal Fmoc group was removed with a 30% solution of piperidine in DMF.

When the full length peptide synthesis was complete, the resin-bound peptide (0.2 mmol) was treated with a solution of trifluoracetic acid (9 ml) anisole (0.2 ml), thioanisole (0.5 ml) and ethanedithiol (0.3 ml) for 2 hr at room temperature. The reaction was filtered directly into Et_2O at 0°C, and stored at 4°C for 4 hr to complete peptide precipitation. The crude peptides were purified to homogeneity by reverse phase HPLC (Delta-Pak C_{18}) using CH_3CN/H_2O (0.1% TFA) as the solvent system (linear solvent gradients: 15–85% CH_3CN for **Leu4**, 5–95% CH_3CN for **Leu6**, 40–95% CH_3CN for **Leu8**). All peptides were characterized by mass spectrometry and amino acid analysis: **Leu4** (PD-MS) 2057.3 (M^+) calculated 2055.6 (amino acid analysis) Leu 4.03 (4) Glx 5.95 (6) Lys 6.02 (6), **Leu6** (PD-MS) 2027.4 (M^+) calculated 2024.6 (amino acid analysis) Leu 6.1 (6) Glx 4.95 (5) Lys 4.95 (5), **Leu8** (PD-MS) 1994.9 (M^+) calculated 1993.6 (amino acid analysis) Leu 8.04 (8) Glx 3.97 (4) Lys 3.99 (4).

Circular dichroism spectroscopy

Spectra were recorded on a Jasco J-600 spectropolarimeter. All spectra were recorded with peptide in a 10 mM phosphate, 100 mM NaCl, pH 7 buffer. All stock solutions were quantitated by amino acid analysis. The spectra were recorded using a 1 mm pathlength cell scanned from 190 to 260 nm. The spectra were an average of three scans with a resolution of 0.2 nm and a scan speed of 10 nm/min. The helical content was calculated from the value of the mean residue ellipticity at 222 nm (Lyu et al., 1991).

Size exclusion chromatography

Size exclusion studies were performed using Sephadex G-50-80 (Sigma) in a 1.6 cm by 90 cm column at 4°C. The eluent (50 mM phosphate, 500 mM NaCl at pH 7) was maintained at a flow rate of 0.3 ml/min and monitored at 214 nm. A standard molecular weight curve was generated using bovine serum albumin, carbonic anhydrase, cytochrome C, and aprotinin. The peptide solutions (0.5 ml, 200 μM) were loaded onto the column, and the apparent molecular weights were determined from interpolation of the standard calibration curve.

Leu4 crosslinking reaction

Leu4-N (4.2mg, 1.8μmol) was combined with an equimolar amount of **Leu4-C** (4.1mg, 1.8μmol) in 1ml of phosphate buffer (100mM, pH 8), and the mixture was stirred in an open flask for 12hr. The crosslinked products were separated by reverse phase HPLC (Vydac C_8, 2.2cm by 25cm, 8ml/min, 30–55% CH_3CN) and analyzed by mass spectroscopy.

Results and discussion

Peptide design

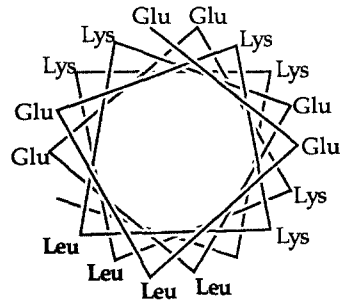
A series of peptides was designed to investigate the components needed to form helical bundles of differing aggregation states. Minimalistic sequences were employed for the peptides which contained amino acid residues with a high potential to exist in an α-helix (Chou and Fasmani, 1974; Scheraga, 1978); either four (**Leu4**), six (**Leu6**) or eight (**Leu8**) leucine residues within a sixteen amino acid sequence (Fig. 1), with the remainder of the residues composed of equal numbers of glutamic acid and lysine residues. The peptide were designed to be amphiphilic with hydrophobic leucines on one face of the helix and the hydrophilic residues on the other. Potential intrahelical salt bridges were designed into each of the peptides (Table 1) by engineering the positioning of glutamate and lysine residues using the program *HELIX* (Chmielewski and Lipton, 1994). The amino terminus of each peptide was acetylated and the carboxy terminus was converted into a primary amide to reduce helix destabilization due to charge repulsion with the helix dipole (Shoemaker et al., 1985, 1987). Also, glutamate was positioned at the amino terminus and lysine at the carboxy terminus in each peptide sequence to increase the potential for charge stabilization with the sidechains of these amino acids and the helix dipole.

Molecular modeling was performed on each peptide to predict potential aggregation states. Since the main driving force for self assembly of amphiphilic molecules in aqueous solution is burying their hydrophobic portions in the interior of the assembly, models were built to minimize the solvent accessible surface isobutyl groups from each leucine residue in the peptides. Based on this criterion it was possible to construct a series of helical bundles with the leucine peptides, whereby the peptide with the smallest exposed continuous hydrophobic surface area, **Leu4** with 450Å, was modeled into a dimer, **Leu6** with 670Å was modeled as a tetramer, and **Leu8** with 900Å was modeled as a hexamer (Fig. 2).

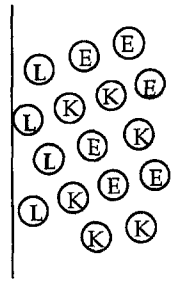
Table 1. Number of potential $i + 3$ and $i + 4$ intrapeptide interactions

	$i + 3$ salt bridge[a]	$i + 4$ salt bridge[a]	$i + 3$ (repulsive)[b]	$i + 4$ (repulsive)[b]
Leu4	4	8	4	–
Leu6	2	6	4	–
Leu8	4	5	–	–

[a] Glu/Lys; [b] Glu/Glu, or Lys/Lys.



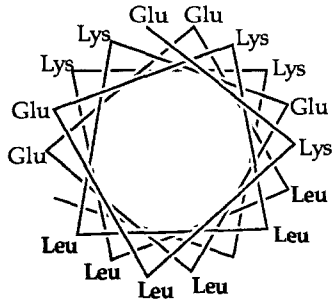
Helical Wheel Representation



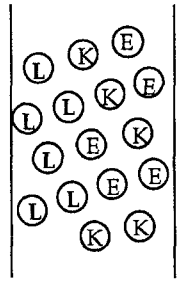
Helical Net Diagram

Leu4 Peptide:

AcNH-Glu-Glu-Leu-Glu-Lys-Lys-Leu-Lys-Glu-Leu-Glu-Glu-Lys-Leu-Lys-Lys-NH_2



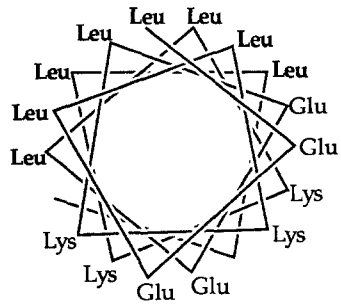
Helical Wheel Representation



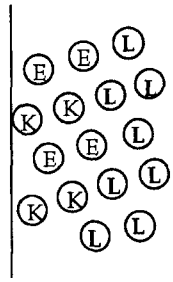
Helical Net Diagram

Leu6 Peptide:

AcNH-Glu-Lys-Leu-Glu-Lys-Leu-Leu-Lys-Glu-Leu-Glu-Glu-Leu-Leu-Lys-Lys-NH_2



Helical Wheel Representation



Helical Net Diagram

Leu8 Peptide:

AcNH-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Leu-NH_2

Fig. 1. Helical wheel and helical net diagrams for **Leu4**, **Leu6**, and **Leu8**

Structural studies

Initial experiments focused on determining if **Leu4**, **Leu6** and **Leu8** existed in helical conformations and if the peptides formed aggregate structures. Circular dichroism (CD) experiments were performed by measuring the mean residue ellipticity between 190 and 260nm at a range of peptide concentrations. At a concentration of 120μM the CD spectra of **Leu4**, **Leu6** and **Leu8** in

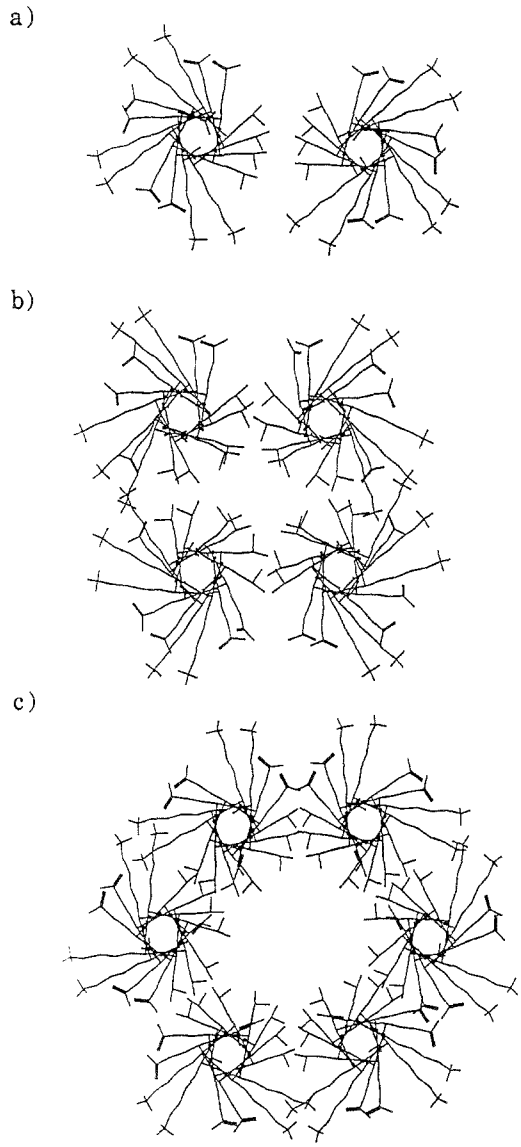


Fig. 2. Molecular modelling of potential aggregation states for **a**) **Leu4**, **b**) **Leu6**, and **c**) **Leu8**

aqueous buffer were typical of α-helical proteins, with helical contents of 77, 90 and 100%, respectively (Fig. 3) (Lyu et al., 1991). As the concentration of the peptides was lowered there was a concomitant decrease in the mean residue ellipticity at 222nm, consistent with the dissociation of a helical bundle into a monomer with a less ordered structure (Fig. 4). The concentration-dependent CD data were analyzed according to various monomer-nmer equilibria using a nonlinear regression program (MLAB) and best fits were obtained for aggregation states of each peptide (Table 2) (Ho and DeGrado, 1987).

The apparent molecular weights of **Leu4**, **Leu6** and **Leu8** were determined by size exclusion (SE) chromatography at 4°C using Sephadex G-50-80. A

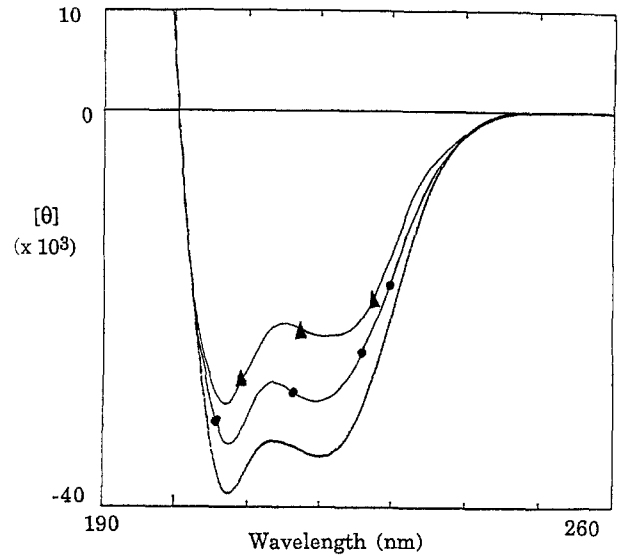


Fig. 3. Circular dichroism spectra for ▲ **Leu4**, • **Leu6**, and — **Leu8**

Table 2. Aggregation states as determined by concentration dependent CD and size exclusion chromatography

	Aggregation state (CD)	Apparent MW (SE)	Aggregation state (SE)
Leu4	2, 3	5,346	2.6
Leu6	4, 5	8,910	4.4
Leu8	6, 8	15,550	7.8

calibration curve was obtained with known protein standards and the apparent molecular weights of the peptides were determined by interpolation of the standard curve. By dividing this value by the monomeric molecular weight aggregation states were obtained for each peptide which are in reasonably good agreement with CD results (Table 2).

*Crosslinking studies with **Leu4***

Within a helical bundle the individual helices may exist in a parallel or antiparallel orientation with respect to the helix dipole. Coiled-coil peptides have been shown to favor a parallel orientation (O'Shea et al., 1991). Comparison between the sequence of **Leu4** and other coiled-coil peptides shows sequence homology in the repeating $i, i + 7$ leucine zipper motif. In an effort to determine the orientation of the helices within the dimer/trimer of **Leu4** crosslinking experiments were performed with modified peptide sequences. Two peptides were synthesized containing the core sequence of **Leu4**, but with a Gly-Gly-Cys sequence added to the N-terminus and a Gly added to the C-terminus (**Leu4-N**), or a Gly-Gly-Gys sequence added to the C-terminus

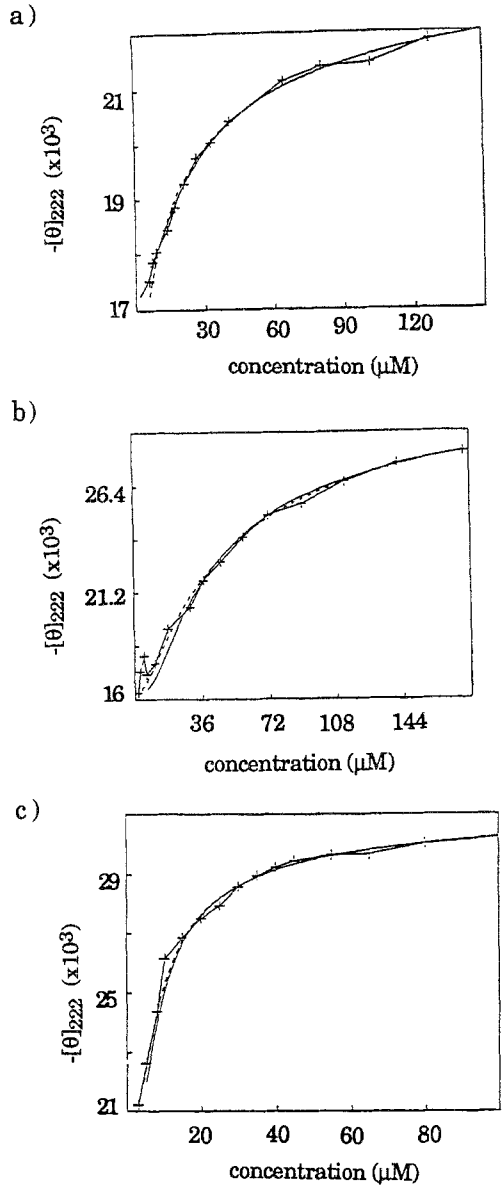


Fig. 4. Concentration dependent helical contents for **a**) **Leu4**, **b**) **Leu6**, and **c**) **Leu8**. The experimental data is indicated with crosses, and curve fitting is shown for **a**) dimer in dashed line, trimer in solid line, **b**) tetramer in solid line, pentamer in dashed line, and **c**) hexamer in dashed line, octamer in solid line

(**Leu4-C**). A 1:1 mixture of **Leu4-N** and **Leu4-C** was equilibrated in aqueous solution, and the free cysteines were air oxidized. If the peptides had no preference for a parallel or antiparallel arrangement a 2:1:1 statistical mixture of peptides **A**, **B**, and **C**, respectively, would be obtained (Fig. 5). Upon oxidation, however, a 1:2:2 mixture of the crosslinked peptides **A**, **B**, and **C** was obtained as determined by HPLC and mass spectrometry, which indicates that approximately 80% of the monomers with a **Leu4** dimer exist in a parallel orientation.

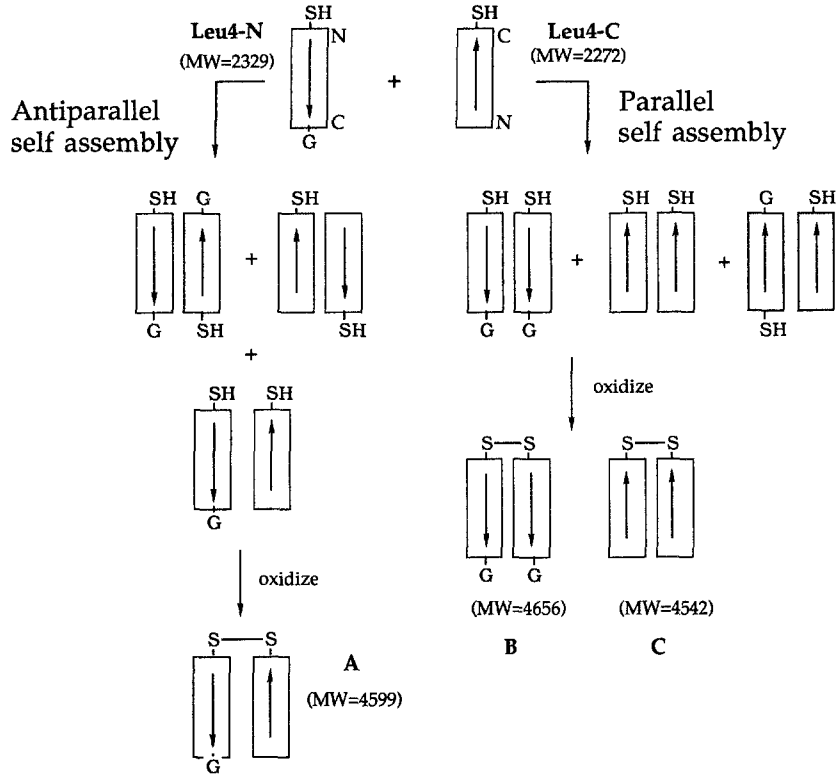


Fig. 5. Schematic of the potential products of **Leu4-N** and **Leu4-C** crosslinking

Conclusion

The peptides of our series were designed to exist in helical conformations and their high helical character was confirmed by CD spectroscopy. The amphiphilic nature of the peptides was designed to insure that differently sized peptide aggregates would be obtained in aqueous solution. All of the peptides exhibited helical contents which were concentration dependent as has been observed with other self-assembling peptides. The size of the peptide aggregates, however, was very dependent on the area of continuous, exposed hydrophobic surface within each of the sixteen amino acid peptides; peptides with larger hydrophobic surface areas formed correspondingly larger helical bundles. Initial modeling experiments predicted that **Leu4**, **Leu6**, and **Leu8** would exists as dimers, tetramers, and hexamers, respectively. The data obtained for these peptides, however, seems to indicate that larger assemblies may be forming with **Leu8**, and also that each of the peptides may exist in more than one aggregation state in solution. It is possible, however, that the shape of a helical bundle is different enough from the globular proteins used as molecular weight standards to make comparison in the size exclusion experiment difficult.

If one compares the exposed continuous surface area in other self-assembling peptides, one finds similar trends between aggregation state and hydrophobic surface area as we have observed. The sixteen amino acid

peptide of Degrado and coworkers (Eisenberg et al., 1986; Ho and DeGrado, 1987) which forms a tetrameric structure has a continuous exposed surface area which is very similar to that observed for **Leu6**, and coiled-coil peptides such as GCN4 have a similar exposed surface area to **Leu4** when the length of the peptide is factored in. The results of Kim and coworkers (Harbury et al., 1994) in which conservative amino acid replacements within coiled coil structures dramatically affected aggregation states are not, however, in accord with evaluations based solely on hydrophobic surface area. In the coiled-coil structure "knobs-in-holes" packing interactions (Crick, 1953) play a significant role in establishing one aggregate form over another. Our research points to definite predictions that may be made on the basis of exposed hydrophobic surface area, but further research is essential to delineate other factors that may be critical for self assembly as well.

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Received August 1, 1995