more hydrophilic ($f = -0.667$) than the $-NO_2$ group ($f = -0.078$), whereas electronically the latter is more electron attracting. Thus if we obtain straight lines by plotting their log k's against the solvent composition Φ, we may expect a crossing of these lines somewhere in between the two limiting Φ values. This expectation is nicely borne out by Figure 4.

Finally, the rate dependence on initial substrate concentration of **16-*p*-NO_2** and **16-*p*-CHO** was studied (Table VI). The log k-log [S] plots are shown in Figure 5. The information provided by these curves has been discussed previously.[17] Suffice it here to conclude on the basis of Figure 5 that the more hydrophobic **16-*p*-NO_2** has a greater tendency to aggregate. It seems amazing that differences in hydrophobicity of a small part of a molecule can manifest themselves so clearly sometimes.

Acknowledgment. We sincerely thank our computer expert Feng Chuanli for carrying out the multiple linear regression analysis.

Induction of Peptide Conformation at Apolar/Water Interfaces. 1. A Study with Model Peptides of Defined Hydrophobic Periodicity

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Abstract: To investigate the role of hydrophobic periodicity in the amino acid sequence of peptides and proteins in determining secondary structure at apolar/water interfaces, isocompositional peptides of leucine (L) and lysine (K) were synthesized. Peptides LKKLLKL (I), $(LKKLLKL)_2$ (II), and LKLKLKL (III) were prepared by solid-phase synthesis with a *p*-nitrobenzophenone oxime ester support. Peptides I and II have hydrophobic repeat periods matching that of an α-helix whereas peptide III has a repeat matching that of a β-sheet. Their conformations in aqueous solution were studied by circular dichroism spectroscopy. Peptide I appears too short to form α-helices as it gave spectra typical of an unordered conformation, while peptides II and III aggregated to form α-helical tetramers and β-sheets, respectively. To further evaluate the effect of apolar/water interfaces on conformation, properties of the peptides at the air/water interface were investigated. Peptides II and III formed much more stable monolayers than I due to their ability to form amphiphilic secondary structures. Transfer of monolayers to solid supports was accomplished by the Langmuir-Blodgett technique. Fourier transform attenuated total reflectance infrared spectroscopy was found to be a powerful technique for examining their conformations. The positions of the amide I and amide II bands observed for peptide II and peptide III were in close agreement with the established values for α-helix and β-sheet structures, respectively. Ultraviolet circular dichroism spectroscopy of the monolayers further confirmed their conformations. These results show that hydrophobic periodicity can determine the structure of peptides and perhaps other polymeric molecules at apolar/water interfaces, offering intriguing possibilities for molecular scale design of surfaces.

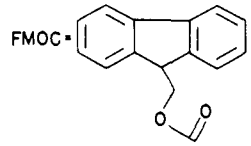
The hydrophobic effect[1] is a prime contributor to the folding and stabilization of protein structures.[2] Hydrophobic residues tend to cluster into the solvent-inaccessible interiors of globular proteins while hydrophilic residues tend to project outward and are more solvated.[3] α-Helices and antiparallel β-sheets often lie along the surface of proteins[4] and are amphiphilic; their solvent-exposed faces are more hydrophilic than their opposite faces which are in contact with the apolar interior of the protein.[5] Amphiphilicity is also important for the stabilization of the secondary structures of peptides and proteins which bind in aqueous solution to extrinsic apolar surfaces including phospholipid membranes,[6,7] air,[6,7] and the hydrophobic binding sites of the regulatory protein, calmodulin.[8] The amino acid sequences of such secondary structures show periodic distributions of hydrophobic and hydrophilic amino acids along the chain[9] with repeat periods corresponding to those of the appropriate structures (e.g., 3.6 for α-helices, 2.0-2.3 for β-sheets). Figure 1 illustrates this concept.

An interesting and important issue is the extent to which hydrophobic periodicity determines secondary structure. Short-range interactions are also known to be important factors in the formation of secondary structures; it has long been known that each amino acid has unique conformational preferences. This conclusion stems from Chou and Fasman's analysis of the different frequencies of occurrence of a given amino acid in specific conformations documented by X-ray crystallography of proteins,[10] studies with amino acid homo- or copolymers,[11] and model building.[12] To investigate the influence of hydrophobic periodicity independent from short-range interactions we synthesized a series of peptides (**1-3**) which contain leucyl and lysyl residues in identical ratios, but with different hydrophobic periodicities.

peptide 1. FMOC $(Leu\text{-}Lys\text{-}Lys\text{-}Leu\text{-}Leu\text{-}Lys\text{-}Leu)_1$

peptide 2. FMOC $(Leu\text{-}Lys\text{-}Lys\text{-}Leu\text{-}Leu\text{-}Lys\text{-}Leu)_2$

peptide 3. FMOC $(Leu\text{-}Lys\text{-}Leu\text{-}Lys\text{-}Leu\text{-}Lys\text{-}Leu)_1$



Chou-Fasman parameters[10] ($\langle P_\alpha \rangle = 1.19$, $\langle P_\beta \rangle = 1.06$) were, of course, identical for all three peptides, as were the Zimm-Bragg s values[11] for helix formation. These peptides differ only in their hydrophobic periodicities and chain lengths allowing the effect

(1) Tanford, C. "The Hydrophobic Effect"; Wiley-Interscience: New York, 1980.
(2) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1-64.
(3) Janin, J. *Nature (London)* **1979**, *277*, 491-492.
(4) Richardson, J. *Adv. Protein Chem.* **1982**, *35*, 167-339.
(5) Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C.; Wilcox, W. *Faraday Symp. Chem. Soc.* **1982**, *17*, 109-120.
(6) Kaiser, E. T.; Kézdy, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 1137-1143.
(7) Osterman, D.; Mora, R.; Kezdy, F. J.; Kaiser, E. T.; Meredith, S. C. *J. Am. Chem. Soc.* **1984**, *106*, 6845-6847.
(8) Cox, J. A.; Comte, M.; Fitton, J. E.; DeGrado, W. F. *J. Biol. Chem.* **1985**, *260*, 2527-2534.
(9) Eisenberg, D.; Weiss, R. M.; Terwilliger, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 140-144.
(10) Chou, P. Y.; Fasman, G. D. *Adv. Enzymol.* **1978**, 47, 45-149.
(11) Scheraga, H. A. *Pure Appl. Chem.* **1978**, *50*, 315-324.
(12) Finkelstein, A. V.; Ptitsyn, O. B. *Biopolymers* **1983**, *22*, 15-25.

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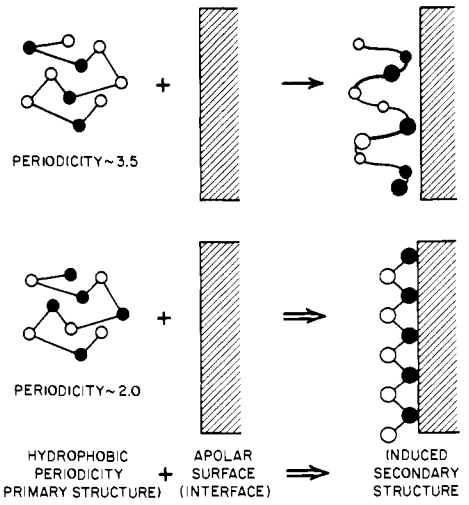


Figure 1. Illustration of the influence of hydrophobic periodicity on secondary structure induction at interfaces. In this highly schematic representation the filled circles represent hydrophobic residues and the open circles hydrophilic residues. In an isotropic environment the chains are in a random conformation, but at an apolar/water interface they adopt repeating conformations which maximize the interactions of the hydrophobic groups with the apolar environments and the hydrophilic groups with the aqueous environment.

of these parameters on peptide conformation to be investigated while maintaining short-range interactions constant.

To investigate the conformational impact of interfaces on these peptides, we measured circular dichroic (CD) spectra of peptide solutions under conditions where aggregation ranged from nil to complete and investigated their monolayer properties at the air/water interface. A wealth of conformational information can be obtained by studying peptide monolayers. Analysis of monolayer surface pressure/area curves gives direct evidence concerning their molecular areas and degree of aggregation.[13,14] Compressed peptide monolayers can be transferred to solid substrates by the Langmuir/Blodgett technique[15] for subsequent conformational determination by infrared[16,17] or circular dichroism[18] spectroscopy. While these thermodynamic and spectroscopic techniques have individually been applied to natural proteins or amino acid polymers, a single comparative study on a well-defined system has not been accomplished. Our peptides of precisely-defined molecular weight and sequence seemed particularly appropriate for this purpose. These studies provide prototypical procedures for analysis of peptide monolayers, as well as showing the importance of hydrophobic periodicity for determining secondary structures.

Experimental Section

Materials and Methods. Peptide syntheses were carried out with a Beckman Model 990B peptide synthesizer programmed as described previously.[20] Amino acid derivatives were purchased from Bachem and were checked by TLC (solvent systems C and D) prior to use. Precoated silica TLC plates (Merck F-254) were purchased through Scientific Products. The following solvent systems were used: A, chloroform/ethanol (98:2); B, chloroform/methanol (95:5); C, chloroform/methanol/acetic acid (85:10:5); D, chloroform/acetic acid (95:5). Spots were detected by UV or by treatment with ninhydrin (0.5% solution in acetone) after exposure to HCl vapors to deblock N-terminal protecting groups. High-performance liquid chromatography (HPLC) was accomplished with a Du Pont Instruments Series 8800 HPLC equipped with a Du Pont Instruments variable wavelength UV spectrophotometer and semipreparative Hamilton PRP1 reversed phase columns (Pierce Chemical Co.).

IR spectra were measured with a Nicolet Series 7000 Fourier Transform infrared spectrophotometer. ZnSe ATIR prisms (45°, 50 × 20 × 2 mm) were from Spectra-Tech or Barnes Analytical. Between uses they were cleaned by plasma discharge for 1 h in a nitrogen atmosphere with use of a Denton Plasma Cleaner with a dry ice trap. CD spectra were recorded with a Jasco Model 500 spectropolarimeter and 0.1–10 mm quartz sample cells. Monolayers for CD measurements were deposited on quartz microscopy cover slips (McCrone Associates) which had previously been washed with soapy water, water, hot nitric acid, and water. UV spectra were recorded with a Hewlett Packard 8450A diode array spectrophotometer. Surface pressure/area measurements and manipulation of monolayers were accomplished with a Lauda film balance equipped with a lifting elevator of our own design. Water was purified by filtration through a Waters Associates Milli Q system. Inorganic salts were from Fisher Analytical and buffers were from Sigma.

FMOCLeu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)-LeuO-*t*-Bu (1A).[19] This peptide was synthesized on a *p*-nitrobenzophenone oxime resin with use of a previously described procedure.[20] Briefly, 5.00 g (3.35 mmol) oxime resin was reacted with 15 mmol each BocLys(2ClZ) and DCC in 60 mL of CH_2Cl_2 at room temperature for 10 h. The resin was filtered and washed with CH_2Cl_2 (4X) and then automatically deprotected and coupled sequentially with the symmetric anhydrides of the appropriate amino acids.[20] The peptide was cleaved from the resin by treatment with 5 mmol of LeuO-*t*-Bu acetate salt[21] in 100 mL of CH_2Cl_2 for 2 days at room temperature. The resulting suspension was filtered and washed 2 times with 70 mL of boiling $CHCl_3$:methanol (9:1) and 3 times with 70 mL of boiling methanol. The solvent was removed under reduced pressure, and the resulting solid crystallized twice from approximately 30 mL of boiling methanol: yield 2.57 g (47%); mp 240–240.5 °C; $[\alpha]^{25}_D$ −24.0° (10 mg/mL in Me_2SO); R_f (A) 0.04, R_f (B) 0.33; amino acid analysis $Leu_{4.05}Lys_{2.95}$. Anal. Calcd for $C_{85}H_{115}N_{10}O_{10}Cl_3$, MW 1639.3, $C_{62.28}H_{7.07}N_{8.54}$; found $C_{62.11}H_{6.94}N_{8.43}$.

FMOCLeu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)LeuOH (1B). **1A** (1.00 g) was dissolved in 10 mL of trifluoroacetic acid/anisol (9:1) and allowed to react at room temperature for 30 min. The solvent was removed under reduced pressure at room temperature and the resulting residue dissolved in methanol and reevaporated at 40 °C. The glassy solid was triturated with ether and crystallized from boiling methanol to remove an impurity which eluted at higher R_f on TLC than the main product: yield 0.87 g (91%); R_f (C) 0.63.

Leu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)LeuO-*t*-Bu (1C). **1A** (1.00 g) was dissolved in 10 mL of dimethylformamide/diethylamine (9:1) and allowed to react 2 h at room temperature. The solvent was removed at 40 °C under reduced pressure and the product triturated with ether: yield 0.86 g (100%); R_f (C) 0.36.

FMOC(Leu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)-Leu)$_2$O-*t*-Bu (2A). **1B** (0.6 mmol), 0.5 mmol of **1C**, and 0.6 mmol of 1-hydroxybenzotriazole hydrate were dissolved in 16 mL of DMF/Me_2SO (3:2) and set on an ice bath. DCC (1.0 mmol) was added and the reaction allowed to proceed on an ice bath for 2 h and at room temperature for 16 h. High performance size exclusion chromatography[22] indicated that the reaction was greater than 90% complete at this time. The apparent pH (wet pH paper) was adjusted to 4 with trifluoroacetic acid and the solvent removed under reduced pressure at 40 °C. The resulting solid was triturated with hot DMF and washed well with DMF and ether: yield 1.15 g (77.4%), homogeneous by criterion of size exclusion HPLC.[22]

(Leu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)-Leu)$_2$O-*t*-Bu (2B). **2A** (120.0 mg) was dissolved in the solvent mixture made up of 5 mL of Me_2SO, 4 mL of DMF, and 1 mL of piperidine and allowed to react at room temperature for 2 h. The solvent was evaporated at 40 °C under reduced pressure and the resulting solid triturated with ether and lyopholized from Me_2SO: yield 106.6 mg (96%).

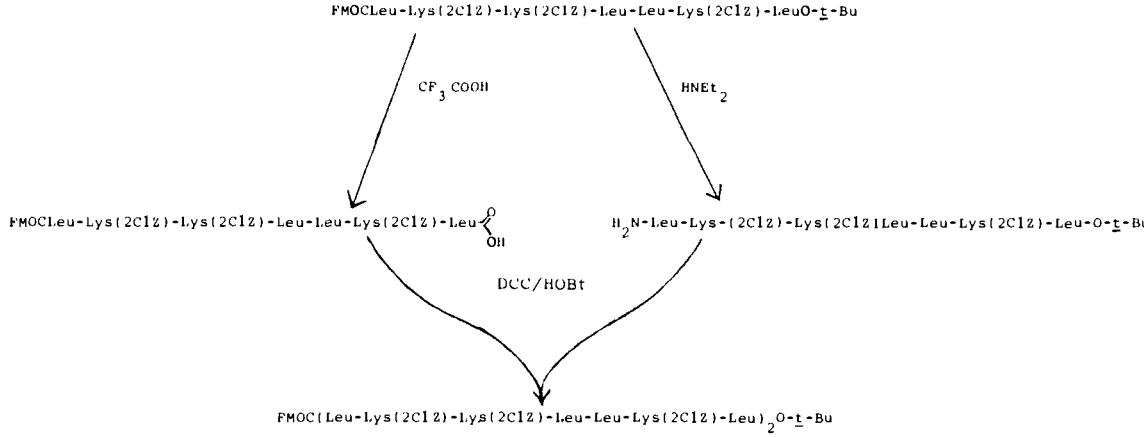
FMOCLeu-Lys(2ClZ)-Leu-Lys(2ClZ)-Leu-Lys(2ClZ)-LeuO-*t*-Bu (3A). This peptide was synthesized from 5.00 g (3.35 mmol) of *p*-nitrobenzophenone oxime resin by the same procedure as for **1A** and crystallized twice from boiling methanol: mp >250 °C; $[\alpha]^{25}_D$ −26.5 (10 mg/mL of Me_2SO); R_f (B) 0.38, R_f (C) 0.73; amino acid analysis $Leu_{4.07}Lys_{2.93}$. Anal. Calcd for $C_{85}H_{115}N_{10}O_{16}Cl_3$, MW 1639.3, $C_{62.28}H_{7.07}N_{8.54}$, found $C_{62.54}H_{6.86}N_{8.22}$.

Leu-Lys(2ClZ)-Leu-Lys(2ClZ)-Leu-Lys(2ClZ)-LeuO-*t*-Bu (3B). This peptide was prepared from **3A** in quantitative yield by the same procedure as for **1C**.

Deprotection of Protected Peptides. Protected peptides were deblocked by reaction with anhydrous HF/anisole (9:1) at 0 °C for 45 min. Typ-

(13) Adamson, A. W. "Physical Chemistry of Surfaces", 3rd ed.; J. Wiley & Sons: New York, 1976; Chapter 2.
(14) Fukushima, D.; Kupferberg, J. P.; Yokoyama, S.; Kroon, D. J.; Kaiser, E. T.; Kezdy, F. J. *J. Am. Chem. Soc.* **1979**, *101*, 3703–3704.
(15) Malcolm, B. R. In "Progress in Surface and Membrane Science"; Danielli, J. F., Rosenberg, M. D., Cadenhead, D. A., Eds.; Academic Press: New York, 1973; Vol. 7, pp 183–229.
(16) Loeb, G.; Baier, R. E. *J. Colloid Interface Sci.* **1968**, *27*, 38–45.
(17) Jakobsen, R. J.; Cornell, D. G. *Appl. Spectrosc.*, in press.
(18) Cornell, D. G. *J. Colloid Interface Sci.* **1979**, *70*, 167–179.
(19) Abbreviations: Boc, *tert*-butyloxycarbonyl; 2ClZ, 2-chlorobenzyloxycarbonyl; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; FMOC, fluorenyl methyloxycarbonyl; *O-t*-Bu, *tert*-butyl ester.
(20) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300.
(21) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261.

Scheme I



ically, 10 mL of HF was used to cleave 0.1–1.0 g of protected peptide. The HF was removed at 0 °C under reduced pressure and the peptide triturated with ether. The products were then dissolved in 50% aqueous acetic acid and purified by HPLC (PRPI semipreparative column, 5–10 mg/injection with a gradient of 10–80% CH_3CN in 0.1 M aqueous trifluoroacetic acid varying at 1%/min) and immediately lyophilized. The products thus purified were greater than 98% pure by criterion of reverse-phase HPLC.

Peptide Monolayers. Peptide monolayers were spread from ca. 100 μM aqueous solutions by expelling drops from the tip of a disposable pipet. Isotherms were measured using a barrier speed of ca. 25 mm/min. Surface pressure/area isotherms were analyzed from 0.2 to 3 mN/m according to the equation[13] of state $\pi(A - A_0) = kT/n\text{DP}$, where π is the observed surface pressure for a given area per amino acid residue (A), DP is the number of residues per peptide (including the FMOC group as a single residue), k is Boltzmann's constant, and T is temperature in degrees Kelvin. Linear regression gives A_0, the limiting molecular area, and n, the degree of association which should be close to unity for an ideal surface gas. The values appeared to be largely independent of the presence of the N-terminal FMOC group.

For spectroscopic studies, peptide monolayers at a constant surface pressure of 20 or 25 mN/m were transferred to quartz cover slips or ZnSe prisms at a rate of 1 mm/min with their faces parallel to the direction of the movement of the film balance barrier and perpendicular to the air/water interface. For deposition of monolayers onto apolar surfaces, the slides were first coated with a monolayer of Cd arachidate. Peptide monolayers were then deposited on these pretreated slides by passing the slide down through the air/water interface and then pulling them out again. The degree of monolayer transfer was monitored by measuring the area change required to maintain constant pressure; both downward and upward movements gave quantitative transfer within 10% experimental uncertainty. For deposition of monolayers onto polar surfaces the monolayers were transferred to untreated quartz slides in a single upward motion. The area change indicated that transfer was quantitative. This was checked by the UV absorption of the strong fluorenyl absorption of the FMOC group; the two methods agreed within 20%. CD spectra free of linear dichroism were measured according to Cornell.[18] Molar ellipticities were calculated from the known molecular areas of the peptides. For infrared measurements a ZnSe prism was first coated with CD arachidate and its spectrum subtracted from that of the same slide after deposition of two peptide monolayers by a downward and then an upward motion.

Results

Synthesis of Peptides 1–3. The protected precursors of these peptides were synthesized on a *p*-nitrobenzophenone oxime resin, a support which is useful for the rapid synthesis of fully protected peptides in multigram quantities.[20,21] Peptides are removed from this support by carboxylic acid catalyzed nucleophilic displacement with amino acid esters acting as nucleophiles. In the present work this resin provides a strategy for the synthesis of peptides protected with the base-labile fluorenylmethyloxycarboxyl (FMOC) protecting groups at the amino terminus, the strong acid-labile 2-chlorocarbobenzoxy group at the side chains, and *tert*-butyl esters at the α-carboxylate. The advantage of this protecting group strategy[23] is that either the amino or carboxyl terminus can be selectively deblocked, allowing the protected peptide FMOC-Leu-Lys(2ClZ)-Lys(2ClZ)-Leu-Leu-Lys(2ClZ)-LeuO-*t*-Bu (**1A**) to serve as a single precursor for the synthesis of the 14-residue peptide **3** (Scheme I). The FMOC or *tert*-butyl groups were removed from portions of **1A** and the resulting peptides coupled in solution in 77% yield by the DCC/hydroxybenzotriazole method.[24] Size exclusion HPLC with a silica column and DMF/TFA (999:1) as eluting solvent[22] proved to be a rapid and efficient method to monitor the coupling reaction as it progressed. Peptides 1–3 were obtained by deprotection of their protected precursors by reaction with anhydrous HF. Reverse-phase HPLC purifications gave 60–80% recoveries based on the amount of crude, deprotected peptide applied to the column.

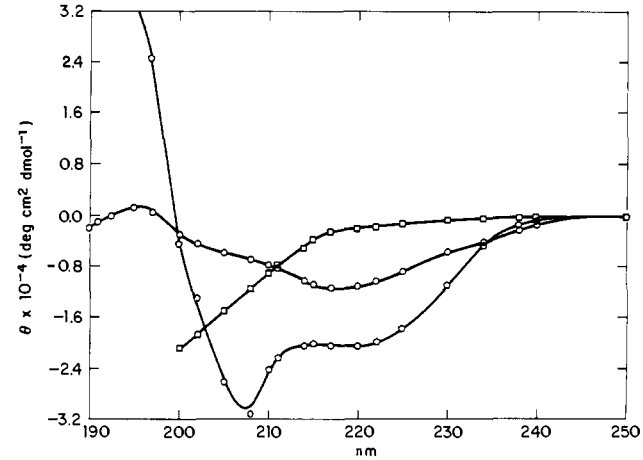


Figure 2. CD spectra of peptides **1–3**. Peptides 1 (□) and 2 (⬠) were dissolved in 0.15 M NaCl, 0.01 M Tris·HCl pH 7.3 to give a peptide concentration of 100 μg/mL. Peptide **3** (○) was 100 μg/mL dissolved in 5 mM Tris·HCl pH 7.3 without added salt because higher ionic strengths led to gradual precipitation. Spectra were recorded with a 1 mm path length cell.

Conformational Properties of Peptides 1-3 in Aqueous Solution. The circular dichroism spectra of aqueous solutions of peptides 1-3 showed pronounced differences (Figure 2). Peptide **1**, at concentrations from 50 to 500 μM in 0.01 M Tris buffer, pH 7.3, containing up to 1 M NaCl gave CD spectra (Figure 2) consistent with a random coil conformation, indicating no tendency for aggregation-induced secondary structure formation. In contrast the spectra for peptide **2** were sharply dependent on both peptide and NaCl concentrations. At low peptide and chloride concentrations, spectra similar to that for peptide **1** were observed, whereas spectra typical of α-helices were obtained at high peptide

(22) Ulyashin, U. V.; Deign, V. I.; Ivanov, V. T.; Ovchinikov, Y. A. *J. Chromatogr.* **1981**, *215*, 263–277.

(23) DeGrado, W. F. In "Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium"; Hruby, J. H., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 195–198.

(24) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788–798.

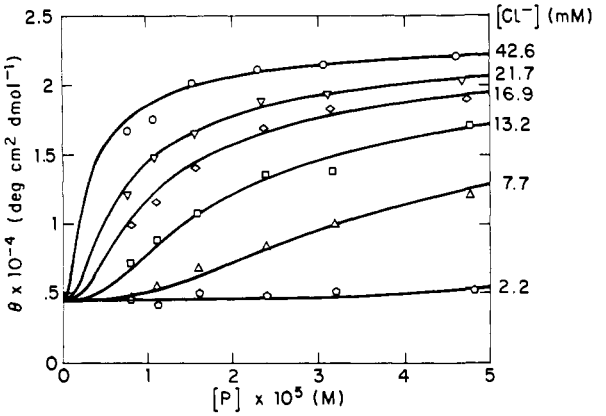


Figure 3. The concentration dependence of the mean residue molar ellipticity at 220 nm as a function of peptide and chloride concentration. The experimental data for a given chloride concentration were analyzed for a cooperative monomer/tetramer equilibrium: $P_{tet} \rightleftharpoons 4P_{mon}$ ($K_{obsd} = [P_{mon}]^4/[P_{tet}]$). The concentrations of the monomeric and tetrameric peptides ($[P_{mon}]$ and $[P_{tet}]$) are given by $[P_{tet}] = ([P_T]/4)(\theta_{obsd} - \theta_{mon})/(\theta_{tet} - \theta_{mon})$ and $[P_{mon}] = [P_T] - 4[P_{tet}]$, respectively, where θ_{mon}, and θ_{tet} are the ellipticities for the monomeric and tetrameric peptides and $[P_T]$ is the total peptide concentration. Combining the above equations and solving for $[P_T]$ gives the equation below. The value of

$$[P_T] = \left(\frac{(\theta_{obsd} - \theta_{mon})K_{obsd}}{4(\theta_{tet} - \theta_{mon})(1 - ((\theta_{obsd} - \theta_{mon})/(\theta_{tet} - \theta_{mon})))^4} \right)^{1/3}$$

θ_{mon} was taken as that found at low chloride and peptide concentrations: θ_{tet} and K_{obsd} were determined from an unconstrained nonlinear least-squares analysis. The values of K_{obsd} depended markedly on the $[Cl^-]$ while the value of θ_{tet} was constant ± 1000 (deg cm²)/dmol. The lines are theoretical curves drawn for $\theta_{mon} = -4500$ (deg cm²)/dmol, $\theta_{tet} = -23800$ (deg cm²)/dmol, and K_{obsd} as determined from the least-squares fit.

Scheme II

$$P_{tet}Cl_m \rightleftharpoons 4P_{mon} + mCl^-$$

$$K_{diss} = \frac{[P_{mon}]^4[Cl^-]^m}{[P_{tet}Cl_m]}$$

and salt concentrations (Figure 2). The spectrum in Figure 2 for this peptide can optimally be described as 70% helix, 30% random coil, and 0% β-sheet, as determined by linear regression with the spectra for α-helical, β-sheet, and random coil forms of polylysine as the independent variables.[25] Alternatively, a similar conclusion can be reached by comparison of our observed value for θ_{220} of -22 000 deg cm² dmol⁻¹ residue ellipticity with the value of -26 800 deg cm² dmol⁻¹ for the helical form of the 13-residue C-peptide from ribonuclease.[26]

The dependence of the coil-to-helix equilibrium on chloride concentration at pH 7.3 is illustrated in Figure 3. At a given chloride concentration, the experimental data may be described by a cooperative monomer–tetramer equilibrium. The data were analyzed as in the figure legend assuming a value of −4500 deg cm² dmol⁻¹ for θ_{220} of the monomer, yielding values of -23 800 ± 1 000 deg cm² dmol⁻¹ for θ_{220} of the tetramer and dissociation constants ranging from 124×10^{-15} to 0.009×10^{-15} M³ for 7.7 and 54.8 mM chloride, respectively. Fitting the data to alternate schemes such as monomer–dimer, monomer–trimer, or monomer–hexamer equilibria gave less satisfactory fits to the data.

The effect of chloride ion in promoting helix formation and aggregation is consistent with the closer proximity of the positively-charged lysyl residues in the tetramer. A simple model to explain this effect involves binding of chloride ions concomitant with tetramerization, as indicated in Scheme II, in which $[P_{mon}]$ and $[P_{tet}]$ are the concentrations of the peptide in its monomeric and tetrameric state and m is the net number of chlorides bound

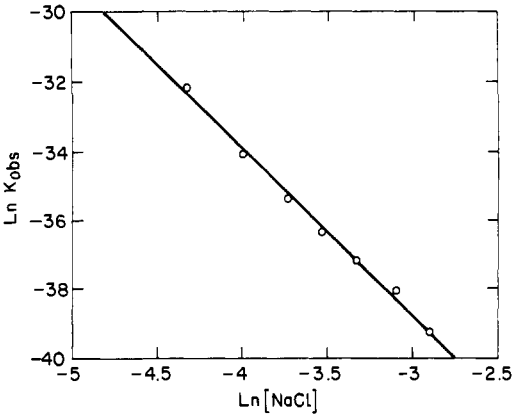


Figure 4. A plot of the natural logarithm of the sodium chloride concentration vs. ln K_{obsd} determined as in Figure 3.

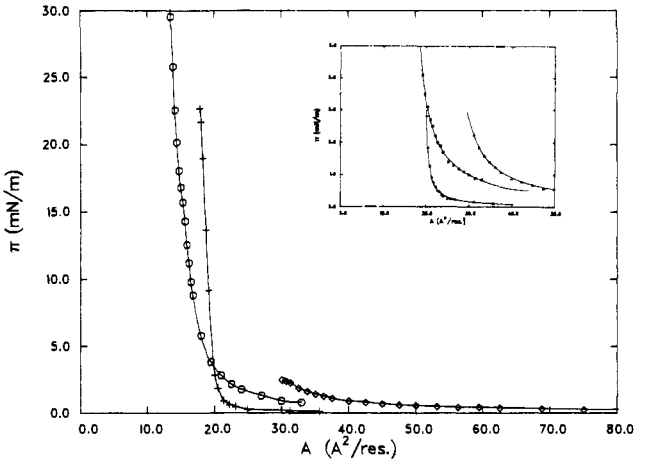


Figure 5. Surface pressure (π) vs. area (A) isotherm for peptides **1** (◇), **2** (○), **3** (+). The peptides were spread from an aqueous solution (peptide concentration 100 μg/mL) onto a subphase of 0.15 M NaCl, 0.05 M Tris·HCl, pH 7.30. The inset shows the 0–4 mN/m region in detail—the lines drawn through the experimental points are theoretical curves generated by using the equation of state described in the Experimental Section and the parameters listed in Table I.

in going from the monomeric to the tetrameric state. This predicts that a plot of the logarithm of the chloride concentration vs. the logarithm of the observed tetramer dissociation constant ($K_{obsd} = [P_{mon}]^4/[P_{tet}Cl_m]$) at that chloride concentration should be linear, giving $-m$ as the slope and $\Delta G_0/RT$ as the intercept (defining the standard state as 1 M NaCl). Indeed, as indicated in Figure 4, an excellent fit (ca. 0.99) is obtained with $m = 4.82$ and $\Delta G_0 = -32$ kcal/mol of tetramer).

Data treatment with use of the more realistic model of Manning[27] which does not require postulating specific ion binding sites gives the same dependence and intercept, but the slope is interpreted as $-1/2$ the number of condensed counterions per tetramer, in this case 10, which need not be associated with specific sites on the tetramer. In either case, the large negative ΔG_0 corresponds to −1.0 kcal/mol of leucyl residues in the tetramer, a value near to the free energy of transfer of a leucyl side chain from water to the less polar interior of a protein (−1.2 kcal/mol, ref 28). This agreement suggests that hydrophobic effects are the major driving force for tetramerization and structure formation.

Peptide **3**, which was expected to form β-sheets upon aggregation, had a strong tendency to slowly precipitate from aqueous solutions, precluding any detailed study of concentration dependences. However, it was possible to dissolve the peptide at 41 μM in 5 mM Tris, pH 7.3, and obtain a solution with stable enough optical clarity to obtain the CD spectrum shown in Figure 2. The

(25) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108–4116.
(26) Labhardt, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 7674–7678.
(27) Manning, G. R. *Q. Rev. Biophys.* **1978**, *11*, 179–246.
(28) Guy, H. R. *Biophys. J.* **1985**, *47*, 61–70.

Table I. Summary of the Analysis of Surface Pressure/Area Isotherms for Peptides **1–3**

peptide	A_0 (A^2/residue)	n (degree of association)
1[a]	ca. 25	
2	16	2.0
2′[b]	17	1.0
3	20	31
3′[b]	21	30

[a] Values for this peptide are approximate due to the fact that monolayers began to dissolve in the subphase at surface pressures as low as 3 mN/m. [b] A prime denotes the absence of an FMOC group.

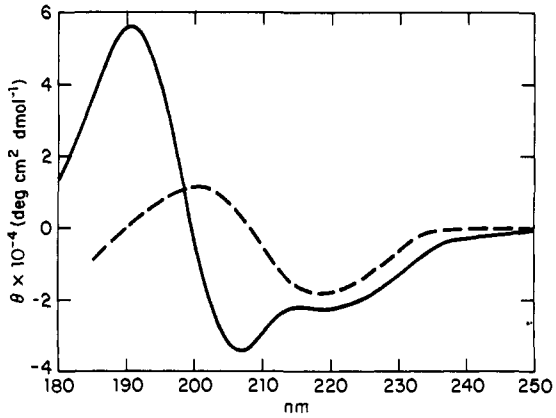


Figure 6. Circular dichroism spectra of transferred peptide monolayers: (—) peptide **2**; (-----) peptide **3**.

broad minimum centered at 218 nm and the maximum near 195 nm are expected for the β-sheet conformation. Analysis with reference spectra as with peptide **2** gave approximately 50% β-sheet character.

The Properties of Monolayers of Peptides 1–3. The above studies suggest that peptides **2** and **3** form amphiphilic secondary structures when they aggregate in aqueous solution. To test whether extrinsic apolar/water interfaces might be able to induce similar conformations in these peptides, the properties of peptides **1–3** at the air/water interface were determined. Figure 5 illustrates surface pressure/area isotherms for peptides **1–3** spread on a subphase of 0.15 M NaCl, 0.05 M Tris·HCl, pH 7.3. As expected from its low tendency to aggregate in aqueous solution, peptide **1** showed marginal surface activity, collapsing at ca. 3 mN/m. In contrast, peptides **2** and **3** formed much more stable monolayers. Analysis of the isotherms (Experimental Section) provided information concerning the molecular cross-sectional areas (A_0) and degrees of association (n) of the peptides (Table I). Peptide **3** is highly associated (n = 31), consistent with it adopting a β-conformation with intermolecular hydrogen-bonding. Peptide **2** was much less associated, consistent with an intramolecularly hydrogen bonded α-helical conformation. The molecular areas of peptides **2** and **3** were reasonable based on an examination of CPK (Corey, Pauling, Kolton) models of the peptides.

To obtain spectral confirmation of the predicted secondary structures of peptides **2** and **3**, monolayers of these peptides were transferred to quartz slides by the Langmuir–Blodgett technique.[13,15] The circular dichroism spectra for monolayers of peptides **2** and **3**, shown in Figure 6, were very similar to their spectra in aqueous solution. Peptide **2** had minima at 222 and 207 nm and a maximum of 192 nm, similar to the position of minima (222 and 208 nm) and maxima (190 nm) found for helices in solution.[25] The magnitude of the ellipticity was the same within experimental error as found in solution. Peptide **3** gave a minimum at 218 nm and a maximum at 198 nm, close to the values of 218 and 195 nm observed for polylysine in the β-form in aqueous solution.[25] The minimum at 218 nm was approximately twice as intense for the peptide in the monolayer as in aqueous solution. However, a detailed quantitative analysis was precluded due to the orientation of the peptides in the monolayers.[18] Monolayers transferred to either polar or apolar surfaces gave qualitatively similar spectra.

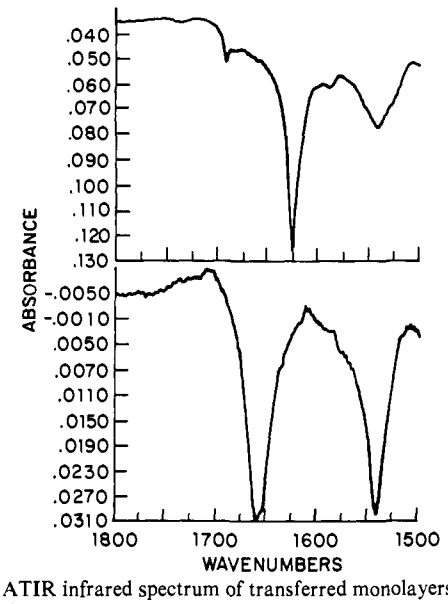


Figure 7. ATIR infrared spectrum of transferred monolayers of peptide **3** (top) and peptide **2** (bottom).

Table II. Infrared Absorption Maxima for Peptides **2** and **3** and for α-Helices and Antiparallel β-Sheets

peptide	amide A	amide I	amide II
peptide **2**	3310	1655	1540
α-helix[a]	3307	1658	1545
peptide **3**	3268	1692, 1626	1540
antiparallel β-sheet[b]	3280	1693, 1624	1534

[a] Reference 30. [b] The value for the antiparallel β-sheet form of Boc(Met)$_7$ immobilized on polyethylene glycol.[31]

Infrared spectroscopy provides an alternate method for determining the conformations of peptides in monolayers. Figure 7 illustrates ATIR spectra for peptides **2** and **3** in the amide I and amide II regions. The positions of these bands, as well as the amide A band (Table II), are in excellent agreement with those expected[30,31] for an α-helix (peptide **2**) or β-sheet (peptide **3**). The amide I region is particularly convenient for assignment of β-sheets, as there is little overlap between the peak near 1630 cm^{-1} for this conformation and the peaks at 1658 and 1665 cm^{-1} for helical and random conformations,[32] respectively. Peaks at 1626 and 1692 cm^{-1} are observed for peptide **3** with no contributions from helical or random structures near 1650 cm^{-1}, indicating that within the limits of this measurement peptide **3** is entirely in a β-sheet conformation. The weak peak at 1692 cm^{-1} is diagnostic of the chains in the sheets being oriented in an antiparallel rather than parallel manner.[33]

Discussion

These results demonstrate that hydrophobic periodicity is an important factor in determining the secondary structures of peptides and proteins. Although peptides **1–3** are composed of the same amino acids in identical ratios, they do not adopt the same conformations. As can be seen in Table III, peptides **2** and **3** aggregate in aqueous solution or bind to the air/water interface, concomitantly adopting secondary structures with repeat periods which match their respective hydrophobic periodicities. The data in Table III also indicate that there are different chain length

(29) Terwilliger, T. G.; Weissman, K.; Eisenberg, D. *Biophys. J.* **1982**, *37*, 353–361.
(30) Rabolt, J. F.; Moore, W. H.; Krimm, S. *Macromolecules* **1977**, *10*, 1065–1074.
(31) Toniolo, C.; Bonora, G. M.; Mutter, M. *Int. J. Biol. Macromolecules* **1979**, *1*, 188–191.
(32) Fawcett, V.; Long, D. A. In "Molecular Spectroscopy"; Barrow, R. F., Long, D. A., Miller, D. J., Eds.; The Chemical Society; Burlington House, London, England, 1973; Vol. 1, Chapter 7.
(33) Moore, W. H.; Krimm, S. *Biopolymers* **1976**, *15*, 2465–2483.

Table III. Hydrophobic Periodicities and Conformations of Peptides **1–3**

peptide	hydrophobic repeat period	conformation in soln	conformation at air/water interface
FMOC(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_1$ (**1**)	3.5	rc[a]	n.d.[b]
FMOC(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_2$ (**2**)	3.5	rc $\rightleftharpoons$ α	α
FMOC(Leu-Lys-Leu-Lys-Leu-Lys-Leu)$_1$ (**3**)	2.0	rc $\rightleftharpoons$ β	β

[a] Random coil. [b] It was not possible to determine due to the low surface activity of this peptide.

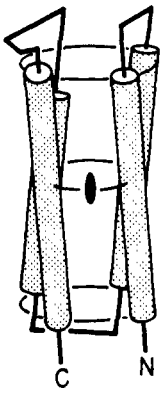


Figure 8. A schematic representation of the folding pattern found in 4-α-helical proteins (taken from ref 37).

requirements for α-helix and β-sheet formation. Helix formation required 14 residues while β-sheet formation was achieved with a peptide of only seven residues. This is in good agreement with previous findings of the critical chain lengths for helix and β-sheet formation.[34] For amino acid homopolymers the critical chain lengths for α-helix and β-sheet formation under forcing conditions (low dielectric solvents which favor secondary structure formation) are approximately thirteen and four residues, respectively. Our results are also in agreement with data obtained with very long copolymers of leucine and lysine (chain length >100 residues). Copolymers in which these amino acids occur in random orders adopt α-helical conformations,[11] but sequential copolymers with repeating leucyl–lysyl dipeptide units form β-sheets.[35] The tendency of random polymers of long chain length to form helices has been well documented.[11] The presence of a hydrophobic period of 2 in the sequential copolymer overrides the short-range conformational preference for helices, causing β-sheet formation. The peptides studied in the present investigation were too short to adopt preferred conformations in dilute homogeneous aqueous solutions, and their conformations at apolar surfaces or in aggregates are determined by hydrophobic periodicity. This can be seen most clearly in the solution behavior of peptide **2**; it is in a random conformation in its monomolecular form, only forming helices when it aggregates.

The consequence of a peptide forming a secondary structure whose repeat period matches its hydrophobic periodicity is that the hydrophobic residues segregate on one side of the structure, forming an apolar surface. This amphiphilic secondary structure can be stabilized relative to other conformations by self-association or binding to extrinsic apolar surfaces, the free energy of dehydration of the hydrophobic side chains being the driving force for the interaction. Natural globular proteins are believed to fold by a similar mechanism, involving hydrophobic interactions between neighboring segments of secondary structures.[5,36] The folding properties of peptide **2** are of particular interest with respect to the folding of natural proteins as this peptide specifically formed tetramers under a wide range of salt concentrations. Tetrameric arrays of α-helices represent a frequently encountered folding motif found in a number of functionally unrelated proteins.[37] In this highly symmetrical fold, the helices pack into a neat bundle with the helical axes of neighboring helices nearly antiparallel to one another (Figure 8), being inclined by just 20°. This angle allows tight packing of the side chains protruding from the apolar side of the helices. We speculate here that the minimal structural requirements for formation of this motif are embodied in peptide **2** and that the tetramers formed by this peptide indeed conform to this folding pattern. Crystallographic investigations are currently underway to investigate this.

A second goal of this investigation was to develop an integrated approach for determining the conformations of peptides at the air/water interface. Previous studies focused on a single technique such as molecular cross-sectional area measurements,[14] IR of collapsed monolayers,[16] or CD of single monolayers.[18] Each of these techniques has associated with it errors and ambiguities: area measurements cannot discriminate between α-helices and β-sheets; CD does not differentiate antiparallel from parallel β-sheets well; and the IR spectra of helical and random structures are similar. Also, spectroscopic studies of Langmuir–Blodgett films are subject to the possibility that the peptides underwent conformational changes during or subsequent to transfer of the monolayer. Only by applying a variety of techniques, including the analysis of force/area curves to determine the degree of association, could the secondary structures of the peptide monolayers be unambiguously assigned. The spectral and thermodynamic properties of peptides **2** and **3** which form simple secondary structures should be useful for analysis of monolayers of more complex peptides and proteins.

The highly-ordered structures of peptide monolayers provide attractive possibilities for the molecular-scale design of surfaces. In particular, β-sheet monolayers are attractive because the regularity of the hydrogen bonds between main-chain amide bonds causes the side chains to project from regular and predictable positions. As long as every other residue is hydrophobic the β-sheet conformation is favored. By varying the side chains which project from the hydrophobic or hydrophilic side of the sheet it might be possible to create monolayers which specifically bind molecules and catalyze reactions. It has been suggested that the β-sheet architecture is appropriate to serve as a template for directed condensation of nucleotides, and an attempt to design such a template is in progress.[38] Applications of β-sheet monolayers need not be limited to catalysts. Recently, there has been considerable interest in the polymerization of amphiphiles in monolayers or bilayers to increase the stability of artificial membranes.[39] Peptide β-sheet monolayers might be considered extreme examples of these polymeric membranes, being stabilized by an infinite series of hydrogen bonds, which are directed perpendicular to the direction of polymerization of the polyamide chains. By increasing the chain length of the hydrophobic residues projecting from one side of the sheet it might be possible to prepare monolayers and bilayers with unprecedented properties. Work along the above lines is in progress.

Acknowledgment. We thank M. Rayner, J. Garrett Forsythe, B. D. Chase, E. Matthews, and H. R. Wolfe, Jr., for their help in this work. We had many useful discussions with D. Cornell, who also sent us preprints of unpublished work. We are also appreciative of M. Buckman for preparation of this manuscript.

(34) Narita, M.; Tomotake, Y.; Isokawa, S.; Matsuzawa, T.; Miyauchi, T. *Macromolecules* **1984**, *17*, 1903–1906.
(35) Brack, A.; Spach, G. *J. Am. Chem. Soc.* **1981**, *103*, 6319–6323.
(36) Ptitsyn, O. B.; Rashin, A. A. *Biophys. Chem.* **1975**, *97*, 175–181.
(37) Weber, P. C.; Salemme, F. R. *Nature (London)* **1980**, *287*, 84–86.
(38) Barbier, B.; Caille, A.; Brack, A. *Biopolymers* **1984**, *23*, 2299–2310.
(39) Fendler, J. *Science* **1984**, *223*, 888–894.