Unusually Stable β-Sheet Formation in an Ionic Self-Complementary Oligopeptide

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SYNOPSIS

A 16-residue amphiphilic oligopeptide (EAK16) with every other residue alanine and also containing glutamic acid and lysine (Ac-NH-AEAEAKAKAEAEAKAK-$CONH_2$) is able to form an unusually stable β-sheet structure. The β-sheet structure is stable at very low concentrations in water and at high temperatures. Various pH changes at 1.5, 3, 7, and 11 had little effect on the stability of the β-sheet structure. The β-sheet structure was not altered significantly even in the presence of 0.1% SDS, 7 molar guanidine hydrochloride, or 8 molar urea. One of the structural characteristics of the EAK16 is its ionic self-complementarity in that ionic bonds and hydrogen bonds between Glu and Lys can form readily between two oligopeptide β-sheet structures. This structural feature is probably one of the factors that promotes its extreme stability. This is the first example of such an extended ionic self-complementarity in a protein structure. EAK16 and its related peptides may have applications as useful biomaterials. It also offers a good model for studying the mechanism of β-sheet formation. Because the oligopeptide can self-assemble to form a membranous structure, it may have relevance to origin of life research. © 1994 John Wiley & Sons, Inc.

INTRODUCTION

Peptides consisting of alternating hydrophilic and hydrophobic amino acid residues have a tendency to adopt a β-sheet structure. It has been reported that poly(Val-Lys), poly(Leu-Lys), poly(Lys-Phe), poly(Tyr-Lys), poly(Glu-Ala), and poly(Glu-Tyr), and the oligopeptides (Val-Glu-Val-Lys)$_{2-3}$ and (Val-Glu-Val-Orn)$_{2-3}$, are able to form stable β-sheets in the presence of salt, various pHs, and prolonged incubation.[1-8] On the other hand, peptides of similar composition without an alternating sequence do not form stable β-sheets; rather they tend to be in random coils or to form a stable α-helix as in one of the examples of poly(Leu-Lys-Lys-Leu).[2] A number of short peptides containing alanine, glutamic acid, and lysine (A/E/K) have been reported to adopt highly stable α-helices in solution.[9-11] Changes in the positions of these amino acids resulted in changes of the helical stability.[9] The helical structures of these peptides are also sensitive to the changes of ionic concentration, temperature, pH, but insensitive to peptide concentration themselves. It was postulated that the helical stability of the peptides are partly due to the ion pairs or salt bridges between the positively charged lysines and the negatively charged glutamic acids. The most stable α-helix peptide in this class also has the strongest interaction potential in the peptide where the lysines and glutamic acids are separated by three alanines with glutamic acid near the N-terminal end $(i + 4)$E,K.[9]

The sequence AEAEAKAKAEAEAKAK was initially found in zuotin, a putative Z-DNA binding protein in yeast.[12] The octomer sequence (AEAEAKAK) is essentially repeated three times in zuotin in a region that is predicted to have a potential α-helix forming segment.[12] In the EAK16 repeats, because there are three amino acids between Glu and Lys as in the case described above [$(i + 4)$E,K], four intramolecular ionic bonds can potentially form in an α-helix—namely, Glu at positions 2 and 4 with Lys at positions 6 and 8 as well as Glu at positions 10 and 12 with Lys at positions

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14 and 16, respectively. However, the CD spectrum of EAK16 in water showed a distinctive β-sheet structure with a minimum at 218 nm and a maximum at 195 nm. The β-sheet structure appears to be stable in very dilute solutions, in contrast to many β-sheet forming peptides whose structures are shown to be concentration dependent.[7,13,14] The EAK16 β-sheet structure is also stable at high temperatures such as 90°C. Furthermore, this structure seems to be not significantly altered at various pHs (1.5, 3, 7, and 11), or in denaturation reagents, e.g., 0.1% SDS, 7*M* guanidine · HCl, or 8*M* urea. Because of the side-chain arrangement in a β-sheet peptide structure, EAK16 is structurally self-complementary on one side of the β-sheet—i.e., they can readily form complementary positive and negative intermolecular ionic bonding and hydrogen bonding; in addition, hydrophobic bonding may be found on the other side of the β-sheet. Interestingly, upon addition of a small quantity of monovalent salt, the peptide spontaneously self-assembles to form a stable macroscopic membranous structure. A brief report of this materilas has been published.[15]

MATERIALS AND METHODS

Peptide Synthesis, Purification, and Determination

The peptide EAK16 was synthesized by solid-phase peptide synthesis on an Applied Biosystems Model 430A peptide synthesizer using standard N-*tert*-butyoxycarbonyl (*t*-Boc) chemistry in cycles using *n*-methylpyrolidone chemistry.[16] Both N- and C-termini of the peptide EAK16 were blocked to resemble the native state of a protein. C-terminal amides were synthesized on *p*-methylbenzhydrylamine resin, while the N-terminus of the peptide was acetylated by using acetic acid anhydride with an equivalent of diisopropylethylamine in dimethylformamide. The peptides were cleaved from the resin using hydrofluoric acid/anisole 10 : 1 (v/v).[17] The peptides were purified through high performance liquid chromatography (HPLC) using a Vydac C_{18} semipreparative column, eluted with a gradient of 5–60% acetonitrile in 0.1% trifluoroacetic acid (TFA), and lyophilized in a speed vacuum. Peptide purity was determined by analytic HPLC and the composition was determined by amino acid analysis. Peptide stock solutions were prepared at a concentration approximately 0.57 m*M* (1 mg/mL) in water (the molecular weight of the EAK16 is 1760). EAK16 has a maximal solubility of 3 m*M* (about 5 mg/mL) in water but can be solubilized up to 6 m*M* (about 10 mg/mL) in 23% acetonitrile. The concentration was determined by ninhydrin methods using internal controls.

CD Measurement

CD spectra were taken on an Aviv model 60DS spectropolarimeter using program 60HDS for data processing. Because EAK16 contains both positively and negatively charged residues, the peptide itself serves as a buffer. CD samples were prepared by diluting stock peptide solution (3 m*M*) either in water, solutions of various pH, different concentrations of NaCl, 7*M* guanidinine · HCl, or 8*M* urea. The experiments determining peptide stability at low concentrations were studied by diluting the peptide solution successively from 20 to 0.61 μM in water. Reversal concentration experiments were also performed—i.e. the lowest concentration of peptide was measured, then more peptide was added. In the pH effect experiments, peptide samples were incubated in different pH buffers 4 h before measurement. Salt effect experiments were performed by incubating EAK16 in NaCl solution from 0.4 to 525 m*M*. In the denaturing experiments, the peptide was mixed with either 0, 1, 3, 4, 5, or 7*M* guanidine · HCl, or 8*M* urea, overnight before CD measurement. In the SDS experiment, SDS was added to the peptide solution for a final concentration of 0.1%. All experiments were performed at 25°C unless otherwise indicated. All reagents used in experiments were ultrapure and solutions were filtered through a 0.22 μm filter.

RESULTS

Stability of the Structure in Dilute Solution

A typical β-sheet CD profile was detected in a very dilute solution of EAK16. In one experiment, a 20 μM EAK16 solution was equilibrated in water and the CD spectrum was then taken. This solution was then serially diluted, equilibrated, and measured five times (Figure 1). At the lowest measurable peptide concentration (0.612 μM), the characteristic of a β-sheet CD spectrum (minimum at 218 nm and maximum at 195 nm) is still clearly recorded (Figure 1a). The CD signal is linearly proportional to the peptide concentration (Figure 1c), but the calculated molar residue ellipticity remains essentially unchanged (Figure 1d), similar to an α-helical forming peptide $(i + 4)$E,K.[9] An isosbestic point was recorded at 205 nm, thus indicating that the structure

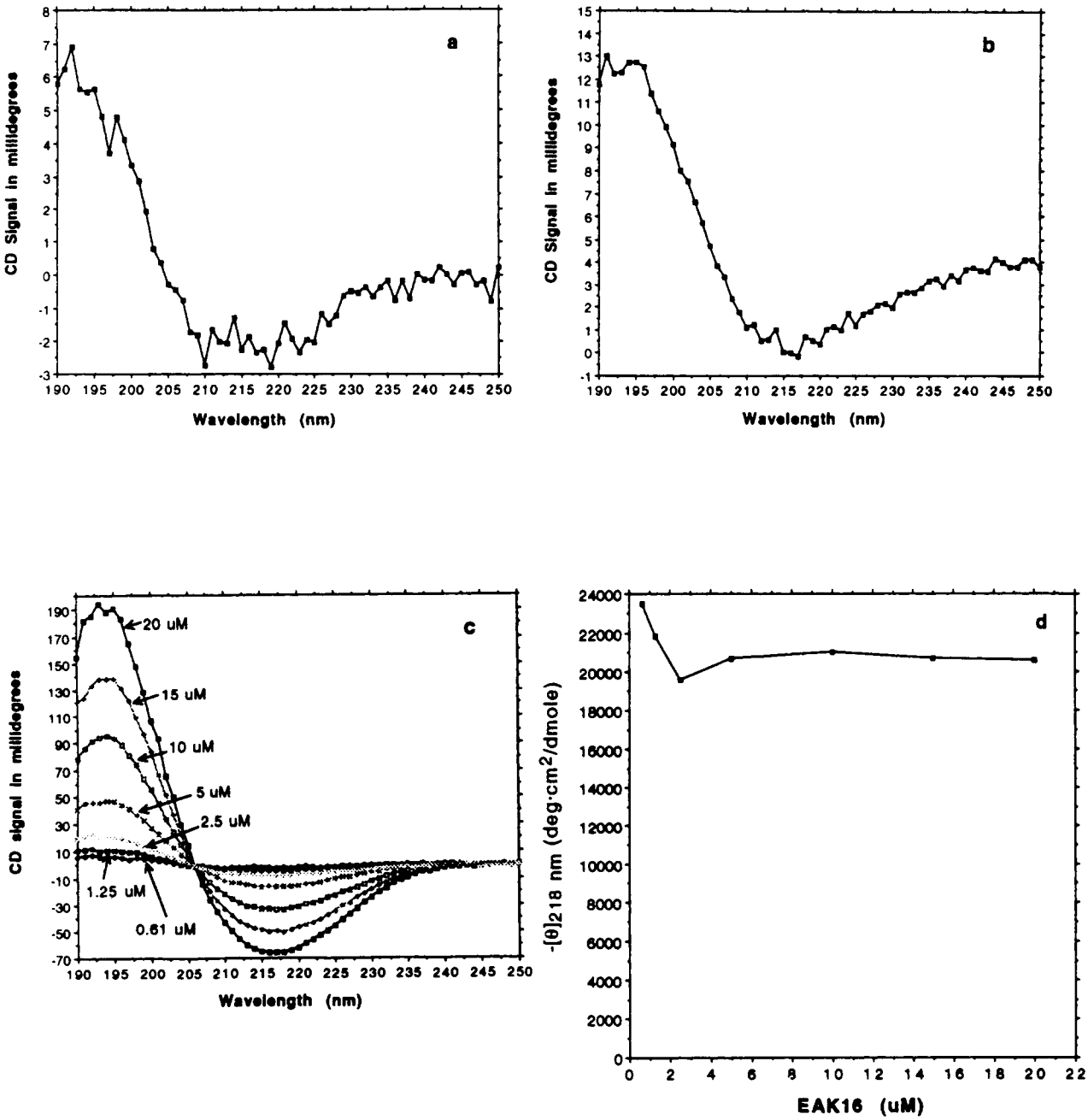

Figure 1. CD spectra of peptide EAK16 in water. The *X* axis is wavelength in nm; the *Y* axis is expressed as CD signal in millidegrees instead of $[\theta]$ in order to show the β-sheet stability of EAK16 at low concentrations. (a) 0.62 μ*M*. (b) 1.25 μ*M*. (c) Concentrations from 0.62 to 20 μ*M* as indicated by arrows (0.625, 1.25, 2.5, 5.0, 10, 15, and 20 μ*M*, respectively). Note that the spectra cross an isosbestic point at 205 nm, thus indicating that the structure has not changed. (d) The plot of normalized peptide concentrations vs the molar mean residue ellipiticity (19) at 218 nm ($-[\theta]_{218nm}$ deg·cm²/decimole).

is stable in these conditions. This suggests that the β-sheet structure is stable even at low concentrations. It is surprising that the EAK16 peptide forms a stable β-sheet in very dilute solution, since other β-sheet forming peptides, such as β(29–42) and β(1–42) of β-amyloid protein[13] and the TL-LRR1 peptide (23 amino acids) from the toll protein in *Drosophila*[14] and others often show a stable β-sheet

only in high concentrations.[13,14] Two possibilities may explain the observations: (1) the CD signal is from single antiparallel β-sheets, or (2) the CD signal is from assemblies of β-sheets stabilized by hydrophobic interactions of alanines and the ionic bonds between lysines and glutamic acids.

Thermal Stability of the EAK16 Peptide

The β-sheet structure of EAK16 is heat stable. Experiments were carried out in the temperature range from 25 to 90°C in water with measurements at 25, 37, 55, 70 and 90°C (Figure 2). The β-sheet thermal stability of EAK16 was also indicated by the ratios of ellipiticity at 195/218 nm, which are approximately 4.0 at all temperatures (Figure 2 and Table I). Furthermore, there is an isosbestic point at 202 nm (Figure 2a). The decrease of the intensity at 218 nm is approximately 20% from 25 to 90°C. This is in comparison to an unusually stable α-helix forming 16-residue A/E/K $(i + 4)$E,K peptides studied previously by Marqusee and Baldwin[9] where the intensity at $-[\theta]_{222nm}$ of $(i + 4)$E,K has a reduction greater than 75% from 0 to 70°C. We have not seen a report of any defined oligopeptide β-sheet structure with such extreme thermal stability.

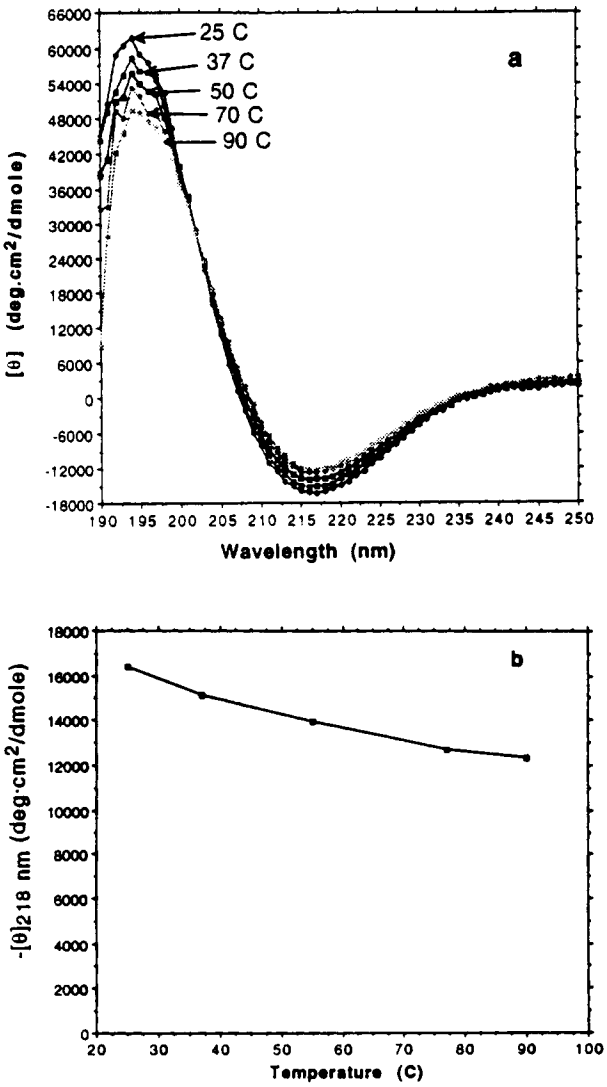

Figure 2. Thermal stability of EAK16. The peptide (8 μM) was measured at 25, 37, 55, 70, and 90°C. (a) The overall CD spectra are shown, the temperatures are indicated by arrows; (b) the thermal profile of EAK16, a 22% decrease of intensity was seen from 25 to 90°C. The oligopeptide solution in water was incubated at 90°C for an hour before taking the spectrum.

Table I Optical Properties of EAK16 at Different Temperatures[a]

Temperature (°C)	$-[\theta_1]_{218nm}$ × 1000	$[\theta_2]_{195nm}$ × 1000	$[\theta_2]/[\theta_1]$
25	16	62	3.9
37	15	60	4.0
55	14	55	3.9
70	13	54	4.1
90	12.5	50	4.0

[a] EAK16 peptide was measured at different temperatures. The minimum and maximum were recorded and the ratio was calculated. The strong stability was suggested by the constant $[\theta_2]/[\theta_1]$ ratio at all temperatures tested. $[\theta]$ = molar minimal residue ellipiticity.

Effect of pH

The β-sheet structure of EAK16 is not significantly affected by pH changes. The EAK16 peptide contains 4 positively charged lysine and 4 negatively charged glutamic acid residues at neutral pH. Lysine has a pKa of 10.0 and glutamic acid has a pKa 4.4 in proteins. EAK16 has a calculated pI of 6.71. It was anticipated that changes of pH would greatly affect the β-sheet structure, especially when the charged groups are neutralized. However, it is surprising to observe only a slight change of the structural stability of EAK16 at all pH values tested, as shown in Figure 3a. All spectra indicate a stable β-sheet structure for the EAK16 from pH 1.5 to pH 11. The difference in ellipticity at 218 nm between each pH point is very small (Figure 3b). This implies that the overall β-sheet structure of EAK16 has not altered significantly at various pHs even though the charged residues, if readily accessible, would have been neutralized for glutamic acid residues. Under

such conditions Glu^- would be neutralized to Glu^0, therefore losing their ionic bonding capability. One the other hand, when pH was increased beyond 12.5, precipitation was observed, similar to that reported for other alternating peptides.[2] This pH stability is also seen in several unusually stable α-helix forming E/A/K based peptides, e.g., the $(i + 4)$E,K motif where glutamic acid and lysine were three residues apart and these ionic side chains were postulated as able to exist as ionic pairs.[9] The slight shift of the minimal ellipticity is probably due to a change from

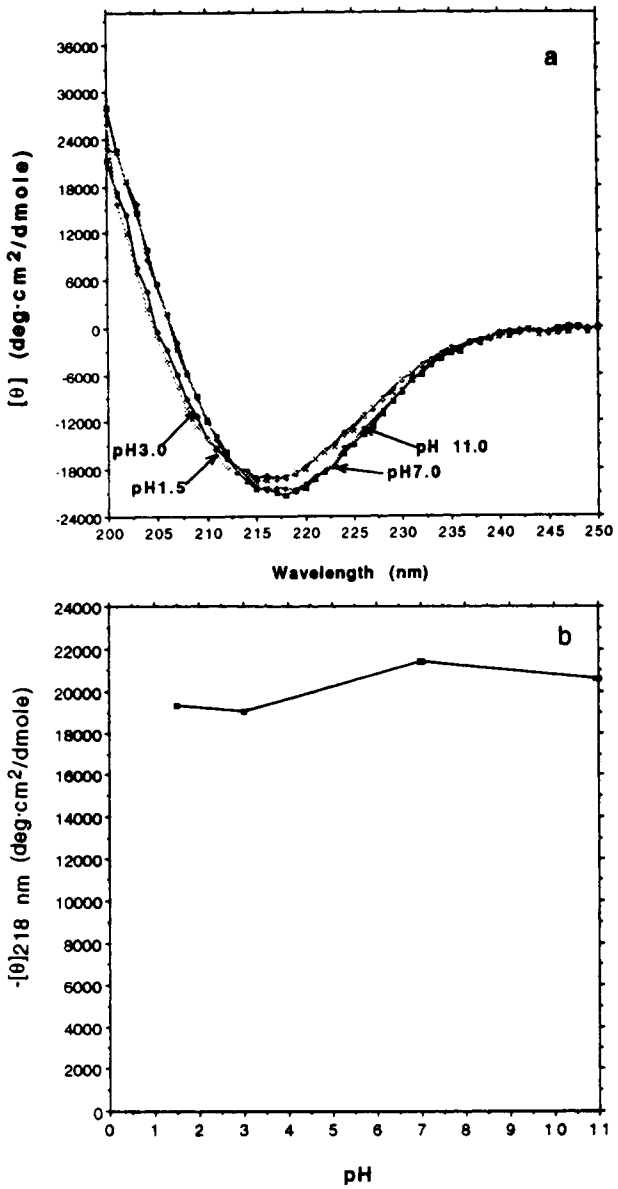

Figure 3. Effect of pH changes on EAK16. (a) The overall CD spectra are shown. (b) The CD pH profile of EAK16. Less than 10% change of $-[\theta]_{218nm}$ deg·cm²/decimole was seen from pH 1.5 to pH 11.

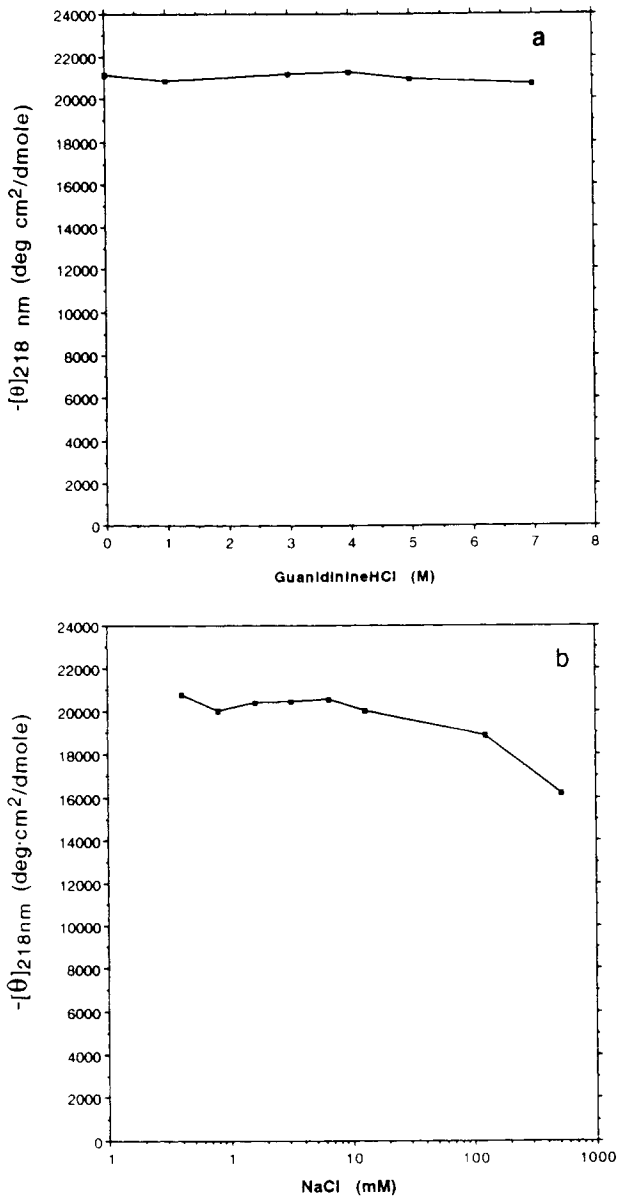

Figure 4. Effects of other reagents on EAK16. (a) Concentrations of guanidinine·HCl vs mean residue ellipiticity $[\theta]$. Urea and SDS gave essentially the same results. (b) The effect of NaCl. The decrease of the $-[\theta]_{218nm}$ deg·cm²/decimole may be primarily due to the aggregated structure[15] as proposed and schematically shown in Figure 5.

side-chain ionic bonding to hydrogen bonding if some charges were neutralized, thus the slight change of the structure (Figure 3a). The structural stability of EAK16 is similar to the β(1–39) and β(1–42) of β-amyloid peptides where the β-sheet structures were essentially unchanged in low and high pH.[13] However, it is in contrast to other β-sheet forming peptides such as poly(Tyr-Lys)[6] and the

TL-LRR1 peptide,[14] which showed pH dependent stability.

Effect of Denaturation Agents

The β-sheet structure of EAK16 is not significantly affected by $7M$ guanidine · HCl and $8M$ urea. Guanidine · HCl and urea are frequently used in denaturing proteins and their aggregates. Figure 4 shows the plot of 218 nm from the CD measurement of EAK16 taken from 0, 1, 3, 4, 5, and $7M$ guanidine · HCl. There are no significant CD signal changes in the concentration range of denaturants up to $7M$ guanidinine · HCl. Similar results are found up to $8M$ urea (data not shown). It has been reported that the concentrations greater than $4M$ of these reagents can effectively denature other β-sheet forming peptides and most proteins.[8] Moreover, 0.1% SDS does not alter the structure significantly either (data not shown). These experimental observations suggest that the EAK16 is forming a strongly stabilized β-sheet structure.

Furthermore, in the presence of monovalent inorganic salts, EAK16 is able to spontaneously assemble into an interwoven filamentous macroscopic membrane structure. These filaments were revealed in a scanning electron microscope study.[15] The reduction of the ellipticity at 218 nm with higher concentration of NaCl (Figure 4b) is perhaps due to the self-association of the EAK16 to form such a membranous structure. This membranous material can be stained by Congo red; thus it is visible to the naked eye.[15]

Other Alternating Oligopeptides

We have also studied the structural properties of several other alternating oligopeptides. These oligopeptides are listed in Table II. Some of these oligopeptides can form a β-sheet structure only in the presence of salt and prolonged incubation. This observation is consistent with previous studies.[1-8] The β-sheet structure of EAK12 is not as stable as EAK16 and it is able to go through a transition between the α-helix and β-sheet (data not shown). It can also form a macroscopic membranous structure with a different texture.[15] On the other hand, EAK8 remained in a random coil conformation even in the presence of salt (data not shown) and it did not form the macroscopic structure. This suggests that the length of the oligopeptide is critical for formation of a stable β-sheet structure.[15] The composition of EAK is similar to the oligopeptides studied by Marqusee and Baldwin.[9,10] However, the oligopeptides in their studies formed an unusually stable α-helix. Thus the type of structure and its stability are de-

Table II Structural Properties of Alternating Amphiphilic Peptides Used in this Study

	Oligopeptide Sequence[a]	Structural Properties			
Name	N → C	Water[b]	Salt Sol'n[c]	Macroscopic[d]	Ref.
EAK16	AEAEAKAKAEAEAKAK[e]	β[f]	β	++++	15
EAK12	AEAKAEAEAKAK[e]	α[f]/β	β	+++	15
EAK8	AEAEAKAK	rc[f]	rc	–	15
(i + 4) E, K	AEAAAKEAAAKEAAAKA[e]	nd[f]	α	nd	9
(i + 4) K, E	AKAAAEKAAAEKAAAEA[e]	nd	α	nd	9
(i + 3) E, K	AEAAKAEAAKAEAAKA[e]	nd	α	nd	9
(i + 3) K, E	AKAAEAAKAAEAKAAE[e]	nd	α	nd	9
RAD16	RARADADARARADADA[e]	β	β	++++	This study
RAD8	RARARDRD	rc	rc	–	This study
VE16	VEVEVEVEVEVEVEVE[e]	rc	β	–	This study
VE24	VEVEVEVEVEVEVEVEVEVEVEVE[e]	rc	β	–	This study
RF6	RFRFRF	rc	rc	–	This study
RF8	RFRFRFRF	rc	rc	–	This study
PJJ74	LELFQPFGTEMERAEVLTGV	rc	rc	–	This study
PJJ72	SDMYSVGVILLELFQPFGTE	rc	rc	–	This study

[a] The one-letter amino acid code is used.
[b] Oligopeptides were dissolved and measured in water.
[c] Oligopeptides were dissolved in water and measured in buffer containing $0.2M$ NaCl.
[d] Formation of macroscopic structures. Plus and minus respectively denote the presence and absence of a macroscopic structure.[15]
[e] Refers to oligopeptides with acetylated N-termini and aminated C-termini. α: α-Helix; β: β-sheet; rc: random coil; nd: not determined.

termined by the primary amino acids sequence rather than the composition. Brack and Caille have also studied polypeptides of identical composition but with different sequences. In the case of poly(Leu-Lys-Lys-Leu), it formed a stable α-helix, while the alternating peptide poly(Leu-Lys) formed a stable β-sheet.[2]

DISCUSSION

Stability of the EAK16 β-Sheet Structure in Dilute Solutions

In general, α-helix formation is concentration independent, as seen in the examples of the $(i+4)$E,K and $(i+3)$E,K oligopeptides,[9] which have a similar composition and identical length to EAK16. It is not surprising that helical structure formation possesses such a property because the helical structure formation is an intramolecular event. On the other hand, stable β-sheet structures of oligopeptides need intermolecular interactions. Intramolecular interaction are rare in β-sheet oligopeptides unless a much a longer polypeptide bends back upon itself, as seen in some proteins. We have not completely ruled out that EAK16 would bend back to form an intramolecular β-sheet structure; however, it seems to be unlikely. When the peptide was purified through a C_{18} column by HPLC, two species of molecules were reproducibly observed from the elution profile. It seems that one is a monomer and the other is a dimer, as estimated by their molecular weight (M. Kelley and R. Cook, unpublished observations). Monomeric molecules of EAK16 in a β-sheet structure would require bending with a loop formation. EAK16, with only 16 residues, may not be able to fold back precisely to form the 4 ionic bonds that could contribute to the intramolecular interaction. Thus, monomeric sheet structure is unlikely to account for the observed stability under such extreme conditions. At a concentration of 0.61 μM EAK16, the lowest concentration measurable by the CD instrument, the spectrum still indicates a typical β-sheet structure. It is possible that even at such a low concentration of EAK16, it has not reached the threshold of disassociation of an intermolecular complex.

An alternative explanation is that as in the case of β-sheet structure, the alternating amino acids on the backbone have relatively strong hydrophobic interactions between the methyl groups of the 8 alanine residues in a manner similar to fibroins. Furthermore, the 8 ionic bonds between the lysine and the glutamic acid residues can interact strongly, as illustrated in the proposed schematic structure (Figure 5), thereby contributing to the stability, even in extreme conditions. It is also likely that the extended oligopeptides in the intermolecular complex are staggered so that the one molecule along its length interacts with several other peptides. This staggered structure may be one basis for such an unusual stability. This notion is supported from the observations that EAK8 (AEAEAKAK) and EAK12 (AEAEAKAKAEAK)[15] do not have such a stable structure because of their shorter length—hence, they are not able to stagger, although they have the similar sequences.

The rate of the β-sheet formation appears to be fast. The β-sheet CD profiles were detected immediately following addition of the peptide in all conditions. This is in sharp contrast with several other β-sheet forming peptides that need hours or days in the presence of salt before a stable β-sheet CD signal can be detected.[1,2]

We are discussing a β-sheet system that may exist in two distinct forms. In one form a single β-pleated sheet (containing many EAK 16 molecules) would be found in the solution. This single sheet has several features that would promote stability. In the middle of a β-sheet, each amino acid residue is held to its neighboring peptides by two hydrogen bonds. However, in the case of EAK16, two other features would promote stability. On one side of the sheet there is a collection of alanine side chains forming a hydrophobic domain that would tend to stabilize the sheet. On the other side of the sheet, there is a number of ionic bonds in which lysine side chains on one peptide strand would be bound to glutamate side chains on an adjoining strand. For the 16 amino acid peptide, there would be 8 ionic bonds holding the strands together. It is the addition of these stabilizing ionic bonds holding the strands together in addition to the hydrogen bonds and hydrophobic interactions that stabilize a single sheet.

However, there is a second stabilization form in which two or several sheets are assembled together in a manner illustrated in Figure 5. In this layered structure, the sheets are held together alternately by hydrophobic interactions and ionic interactions between the sheets as well as the ionic interactions within the sheet that stabilize the single sheet itself.

At present, we do not know whether the stabilized β-sheet found in aqueous solution consists of only a single stabilized β-sheet or a small number of β-sheets stacked together, as in Figure 5. What is clear is that the addition of small amounts of cations greatly accelerates the aggregation of β-sheets to

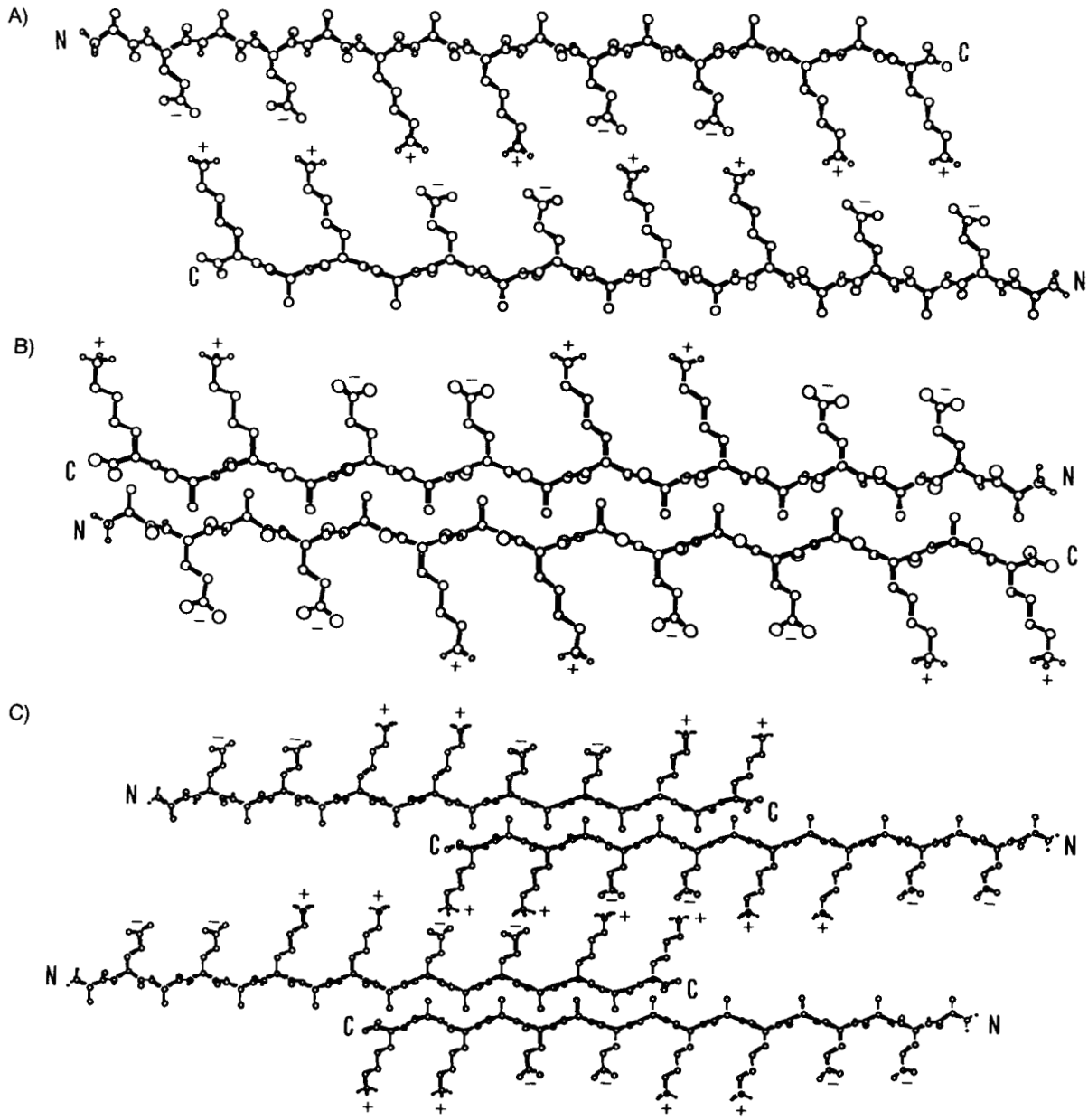

Figure 5. A proposed schematic model of the interactive EAK16 structure. (A) Two EAK16 peptides are shown here viewed so that the β-sheet is horizontal. Both antiparallel and parallel conformations are possible but only antiparallel is drawn here. N and C denote the N- and C-termini of EAK16. The longer side chains are lysines with a plus, and the short ones are glutamic acids with a minus. The pairings of ionic bonds on the side chains are emphasized. When carboxylic groups of glutamic acids are protonated at low pH, a single ionic bond may be changed to become two hydrogen bonds (not drawn). (B) Hydrophobic interactions of alanine residues. The methyl groups are represented by a small circle. Similar interactions are found in silk fibroin. (C) Four EAK16 peptides are staggered along their length. The staggered molecules could be extended in the horizontal direction. (D) A side view of the peptides where the conventional hydrogen bonds on the peptide backbones are formed as indicated by braces. The view is down the β-sheet. The alanines of peptides 1–2 and 3–4 are held together by hydrophobic interactions and the side chains of lysine and glutamic acids of peptide 3–4 and 5–6 form complementary ionic bonds or hydrogen bonds depending upon pH conditions. Hydrophobic interactions are labeled *a* and *b*. The longer side chains are positively charged lysines (a plus), while the shorter ones are negatively charged glutamic acids (a minus). The region of complementary ionic interactions are labeled *c* and *d*. In this diagram the distance between the upper and lower set of side chains is enlarged for clarity.

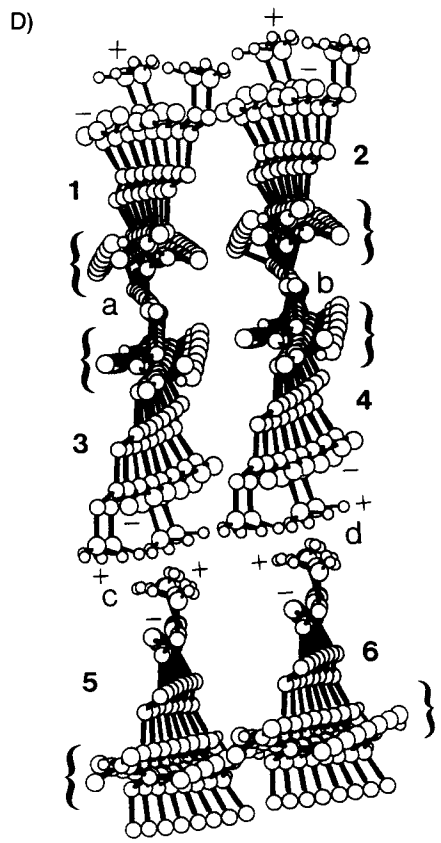

Figure 5. (Continued from the previous page.)

form what eventually becomes a stable macroscopic structure.

Stability of the EAK16 Peptide Under Other Conditions

The β-sheet structure of EAK16 peptide is stable at high temperature. The stability is probably largely due to strong intermolecular interactions. Heat-stable and heat-inducible β-sheet peptides have been reported. In the case of poly(Leu-Glu) and poly(Leu-Lys), a transition to the β-sheet from the random coil or α-helix was observed by heating at 70°C in the presence of salt.[2] It is postulated that peptides in a less stable form have mostly intramolecular interactions of random coil and α-helix. During heating, the less stable forms are denatured and in turn they then tend to favor more stable β-sheet intermolecular interactions. For EAK16, no clear transition of thermal denaturation was observed from 25 to 90°C. This stability is consistent with the ratio of the ellipticity at both 195 and 218 nm, which has essentially a value of 4.0. The approximate 20% reduction of ellipticity at 90°C could be due to the partial denaturation of the hydrogen bonding on the β-sheet backbone.

EAK16 is stable in various pH. This stability is likely to also be due to the complementary ionic bonds or hydrogen bonds between Glu and Lys when EAK16 exists in the β-sheet form either as a single sheet or in an intermolecular β-sheet complex (Figure 5).[9] The ionic complementary interactions can form strong hydrogen bonds. Although the carboxylic groups of glutamic acids have been protonated at pH 1.5 and 3.0 if they are accessible, they still are capable of forming hydrogen bonds as hydrogen acceptors with the hydrogens from protonated amino groups on the side chains of lysines. Similar examples were seen in oligopeptides $(i + 4)$E,K and $(i + 4)$K,E where a change of pH had little effect on their stability.[9] Furthermore, EAK16 is stable in denaturing agents as high as 7 *M* guanidine · HCl and 8 *M* urea. This peculiar kind of stability was not expected. One of the structural characteristics of the EAK16 is its self-complementarity where both ionic bonds and hydrogen bonds between Glu and Lys can form readily between two oligopeptides strands (Figure 5a) and they can also have stabilizing hydrophobic interactions (Figure 5b). This structural property is probably one of the factors determining the extreme stability. This is the first example of such an extended self-complementary ionic structure in peptide or protein structures. Since the β-sheets are staggered and overlayed (Figure 5c), the collective interaction energy is much greater than for individual β-sheet. It appears that even in extreme conditions, the stabilization energy is much greater than that the disruption energy.

It is interesting to note that EAK8 (AEAEAKAK) and EAK12 (AEAKAEAEAKAK) peptides do not have such unusual stability. EAK8 exhibited a random coil structure under identical experimental conditions. EAK12 appears to denature from a β-sheet at high temperature, and it subsequently undergoes reversible helix–coil transitions. These observations suggest that the length is important for such an unusual β-sheet stability in addition to the sequence complementarity. EAK12 is not completely self-complementary. This phenomenon resembles the property of oligonucleotides where their length and complementary pairing are key factors for their stability.

TL-LRR1, a peptide of 23 amino acids with many hydrophobic residues from a leucine-rich repeat was found in Toll protein in *Drosophila*. This peptide exhibited a β-sheet CD spectra and was able to aggregate to form filaments of 7–8 nm in width and several micrometers in length.[14] However, unlike the

EAK16 peptide, formation of such a β-sheet structure of TL-LRR1 is concentration dependent and the structure is sensitive to pH changes. Furthermore, various lengths of peptide segments β(29–42) and β(1–42) of β-amyloid proteins also exhibited β-sheet CD spectra. Again, the β-sheet structure of β(29–42) is sensitive to its concentration.[13] Likewise, these peptides can self-aggregate in the presence of salt to form filaments.[13] Other alternating hydrophobic/hydrophilic peptides have also been reported to form β-sheets and they often show pH and time-dependent β-sheet formation.[1–7]

The unusual stability of the EAK16 β-sheet has some interesting implications for possible roles in prebiotic evolution. In the case of EAK16, because of its self-complementarity, it is able to form a membranous structure in the presence of millimolar concentrations of monovalent inorganic salt. The membrane is very stable and resistant to 10% SDS disruption and it resists degradation by several proteases.[15] It is possible that short peptides such as EAK16 might be condensed in the prebiotic soup from amino acids and short peptides. They would then be preserved and selected because of their stability.

Peptides with positively charged side chains have been reported as able to enhance efficiently hydrolysis of oligoribonucleotides. Poly(Leu-Lys) and poly(Leu-Arg) have a rate enhancement of 185- and 74 fold, respectively, in comparison with controls. They have a 2–5-fold enhancement over poly-(Lys).[18] EAK16 has also showed partial nucleotide hydrolytic activity. It is not surprising that EAK16 has this activity because the side chains of lysines and glutamic acids may be capable of performing general acid–base catalysis.

The mechanism of β-sheet formation is not very clear at the present time. One reason is that there has not been a good model system to study this process. In general, synthetic peptides that form β-sheets tend to aggregate and to produce insoluble materials that form large structures comprising many peptides. Few of the β-sheet forming peptides are highly soluble; in addition, intramolecular structures are formed only at very high dilutions. Therefore it is presumed that the kinetics of β-sheet formation is first order, but the rates are very slow.[19] Based on our study, it seems that self-complementary EAK16 peptides and their derivatives offer a good model system for the study of the mechanism of β-sheet formation.

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