Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 7-13

FORMATION OF AN EXTREMELY STABLE POLYALANINE β-SHEET MACROMOLECULE

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Received April 19, 1995

We have designed a 16-mer peptide composed of a stretch of alanine residues (Ac-$KA_{14}K$-NH_2) which is an effective, simple model for the study of β-sheet formation in the hydrophobic cores of proteins. This peptide adopts an aqueous soluble "bundling" macromolecular β-sheet structure, which is extremely stable to a wide range of pHs, temperatures and/or denaturants. Its unusual stability appears to be due to tight hydrophobic packing of the alanine residues in multilayer sheets or micellar forms with the multimeric lysine array being directed outward at the aqueous environment, allowing aqueous solubility.

The nature of the driving force(s) behind β-sheet formation remains unclear. However, it is likely that this driving force is due either to hydrophobic collapses or hydrogen bonding within the structural elements of neighboring strands. A simple model to study such phenomena is not available since most β-sheet structures not only form complex aggregates, but are also highly insoluble in aqueous solution. A medically devastating example of this is the formation of β-amyloid during the course of Alzheimer's disease (1). An improved understanding of the driving forces causing highly stable β-sheet formation would further our understanding of the nature of the interactions occurring between biological molecules and the hydrophobic cores of proteins. Recently, a stable β-sheet structure has been reported for an alanine-based peptide containing a combination of alanine/glutamic acid/lysine residues (2). This β-sheet structure was proposed to be stabilized by strong hydrophobic interactions through the methyl groups of alanine on one side of the sheet, and by eight ionic bonds between lysine and glutamic acids at *i+2* positions on the other side of the sheet. Interestingly, alanine-based peptides of similar compositions, but having different primary sequences, have been reported to fold into stable, monomeric α-helices in

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0006-291X/95 $5.00

aqueous environments (3). The α-helical conformation found was, in these cases, stabilized by the formation of salt bridges between the lysine and glutamic acid residues at the *i+4* positions. These two separate reports indicate that the nature of the structure of such peptides, as well as their stabilities, are driven by primary structure considerations which are independent from their composition.

The ability of multiple alanine residues to form stable β-sheet structures was studied in the work reported here using a 16-residue peptide composed of 14 alanines, with lysines at both termini to permit aqueous solubility (Ac-$KA_{14}K$-NH_2, named KAK). It should be noted that β-sheet structures were found to occur during the solid phase synthesis of alanine stretches (4-6). Not only does KAK adopt a β-sheet structure in an aqueous environment, but this structure was also found to aggregate in a macromolecular packing mode via intermolecular hydrophobic interactions. The occurrence, as well as the stability, of this β-sheet macrostructure was studied under different environments. The insertion of a single proline in the alanine stretch (Ac-KA_8PA_5K-NH_2, named KAPK) resulted in the complete inhibition of the β-sheet macrostructure formation. The formation of highly stable secondary structures such as the β-sheet macrostructure reported here furthers our understanding of protein interiors, as well as the stability of those enzymes that function at extreme conditions of pH, temperature and/or the presence of organic solvents or denaturants.

MATERIALS AND METHODS

The peptides were prepared by simultaneous multiple peptide synthesis (SMPS) using p-methylbenzhydrylamine resin and standard Boc chemistry (7). Final cleavage and deprotection were carried out using a "low-high" HF protocol (8). Peptides were extracted with water and purified by preparative RP-HPLC. Analytical RP- HPLC was carried out using a Vydac C_{18} column (ODS, 3μ, 5cm x 4.6mm), and monitored at 214nm. The mobile phase consisted of 0.05% TFA in H_2O (solvent A, pH 2.5) and 0.05% TFA in acetonitrile (ACN - solvent B). The peptides were characterized by laser desorption time-of-flight mass spectroscopy analyses (Kratos Kompact Maldi-Tof mass spectrometer). Size exclusion chromatography was performed using a Beckman SEC 3000 column with 60% ACN/ 0.05% TFA/ H_2O as the mobile phase.

All CD measurements were carried out at 25° C on a Jasco-720 CD spectropolarimeter equipped with a Neslab RTE110 temperature controller. A stock solution of each peptide in distilled water was prepared and the exact concentration of the peptide was determined by quantitative amino acid analysis (Multiple Peptide Systems, San Diego, CA). Stock solutions of MOPS buffer (5mM, pH=7.0), 10M urea and 10mM SDS in water were prepared. All measurements were made in 1 or 10mm path length quartz cells. CD results are shown either as CD intensity in mdeg or mean residue ellipticity [θ] in $degcm^2dmol^{-1}$.

Peptide digestion was monitored by C_{18} RP-HPLC using a 0.14μg/ml solution of protease K and a 200μM solution of peptide in phosphate-buffered saline (PBS - 35mM phosphate buffer, 0.15M NaCl, pH 7.0).

RESULTS AND DISCUSSION

Following its synthesis, crude KAK was found to be composed of two distinct populations (Fig. 1A). The major component was represented by a broad peak centered at 3.2min in RP-HPLC, and a second sharp peak at 2.5min. The two peaks, which were separated and purified, showed similar molecular weights by mass spectral analysis (1310.9 and 1311.8 daltons for the broad and sharp peak, respectively, theoretical MW: 1309.7 daltons). If one assumes from the mass spectral results that only a single linear sequence has been generated, then the presence of two distinct secondary or tertiary structures must be responsible for the RP-HPLC results. In 5mM MOPS buffer, the population corresponding to the broad peak was found by CD to exist in a β-sheet conformation (Fig.2A), while the sharp peak fraction adopted a partial α-helical conformation (results not shown). In an initial study, further analyses were carried out on the peak having a β-sheet conformation. This material is referred to as the KAK peptide in the following discussion. The insertion of a proline residue in the alanine stretch (peptide KAPK) resulted in a peptide lacking a β-sheet population. Thus, for KAPK, a single sharp peak at 1.96min was observed by RP-HPLC (Fig.1B). This peptide was found to be random in 5mM MOPS (Fig.2A). This absence of β-sheet structure is consistent with early statistical work which

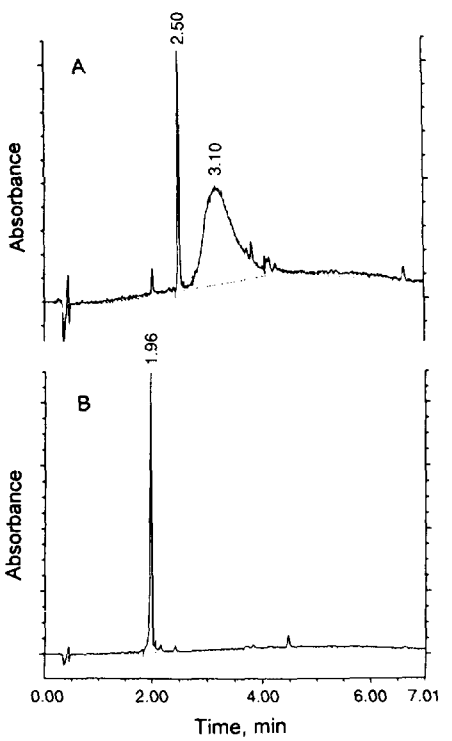

Figure 1. **RP-HPLC chromatograms of (A) KAK and (B) KAPK.** A 12% gradient per minute starting at 5% B; a flow rate of 2ml/min was used.

assigned a β-sheet conformational parameter of P_β=0.62 to proline [i.e., β-sheet breaker residue - (9)]. This β-sheet disrupting effect may be explained either by the presence of the pyrrolidine ring, which can be expected to perturb efficient hydrophobic contacts, thus effectively preventing packing of the β-sheets against one another, or by the lack of an amide hydrogen in proline which is required for hydrogen bonding interactions.

The β-sheet structure of KAK was found to be concentration-independent and was retained at very low peptide concentrations (1μM - Fig.2B). This suggests that a highly stable β-sheet complex has formed, which may be driven by an efficient hydrophobic packing of multiple β-sheets against one another. The stability of the β-sheet structure was further investigated in hydrophobic-like environments such as SDS (10,11), and in α-helical promoting environments such as TFE (12). The β-sheet conformation was retained upon the addition of 7mM SDS, while no effect on the β-sheet was also observed in the presence of 80% TFE (Fig. 3). These results further demonstrate the extraordinary stability of KAK's β-sheet nature. In contrast, the proline containing peptide (KAPK) adopted a partial α-helical conformation in 80% TFE (Fig.3). Interestingly, a similar α-helical conformation was also observed for KAPK in the presence of SDS (Fig.3). Although the role of SDS in promoting α-helicity is not clear, it is conceivable that, in the case of KAPK, SDS stabilizes the α-helix by means of electrostatic interactions with the lysine residues, as well as by providing the appropriate hydrophobic interactions with the alanine

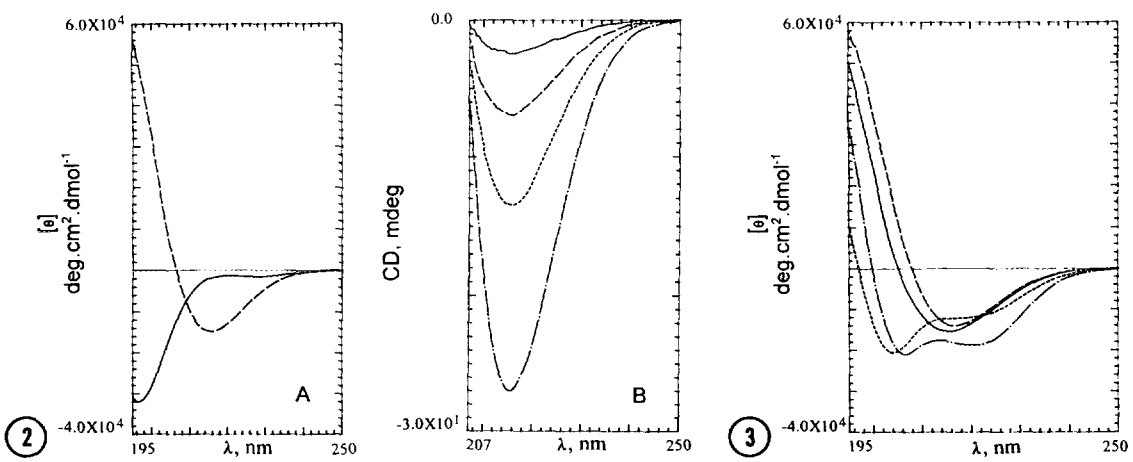

Figure 2. **CD spectra in aqueous solution.** The CD spectra were measured in 5mM MOPS at 25°C. (A) The mean residue ellipticities [θ] of 100μM solutions of KAK (———) and KAPK (——) are shown. (B) Concentration-dependent CD spectra of KAK at a peptide concentration of 1μM (——), 2.5μM (———), 5μM (·····), and 10μM (—·—).

Figure 3. **CD spectra of KAK and KAPK in the presence of TFE and SDS.** The CD spectra were measured in 5mM MOPS at 25°C in the presence of 80% TFE, KAK (——) and KAPK (·····); or 7mM SDS, KAK (———) and KAPK (—·—).

side chains. This was also found in an earlier study of α-helical induction by SDS observed for amphipathic $(Leu\text{-}Lys)_n$ peptides (11).

The conformational stability of the β-sheet structure was further evaluated as a function of temperature or pH. Thermal stability was studied by monitoring the CD spectrum of KAK from 5 to 85° C (Fig.4A). Elevated temperature is known to reduce hydrogen bonding and, in turn, to destabilize most secondary structures. The β-sheet structure of KAK was completely retained at the temperature range studied. These results strongly suggest that hydrophobic interactions are the primary driving force involved in the observed β-sheet complexes. Complete stability was also observed for KAK at pHs ranging from 2 to 10.5 (Fig.4B). Due to the basic nature of the peptide, pHs greater than 10.5 resulted in peptide precipitation caused by neutralization of the protonated lysines. The stability to pH indicates that the charges of the lysine residues, while contributing to the solubility of the peptide, do not interfere with the formation of the β-sheet structure.

Protein stability can be evaluated following the measurement of the secondary structural content in the presence of urea (13). Urea is thought to denature peptides and proteins by providing hydrogen bonding partners for carbonyls and amide hydrogens, resulting in the destabilization of secondary structures. If hydrophobic interactions, more than hydrogen bonding, are responsible for KAK's β-sheet structure stability, then one would expect little or no effect on the secondary structure of KAK by urea. Exposure of KAK to 1 to 8 M urea was found to have

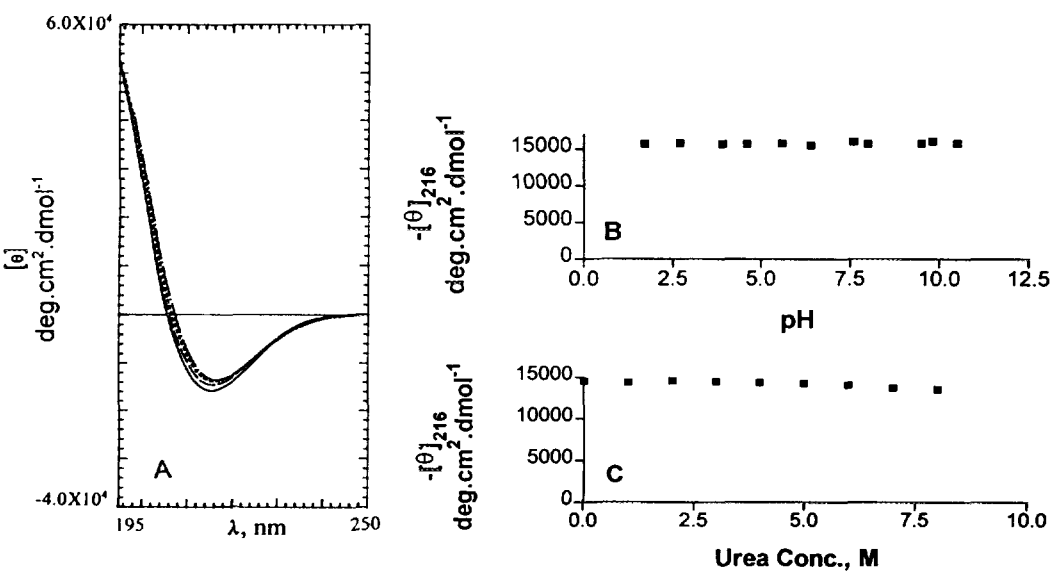

Figure 4. **Effect of temperature, pH and urea on KAK.** (A) CD spectra of KAK in 5mM MOPS at 5° C (——), 30° C (— — —), 60° C (·····), and 85° C (·—·—·). (B) The mean residue ellipticity at 216nm ($[\theta]_{216}$) is plotted as a function of pH, or (C) of urea concentration.

no detectable effect on its β-sheet content (Fig.4C). KAK was also found to be stable to enzymatic degradation in the presence of protease K in PBS buffer at pH 7.1 (Fig.5). Resistance toward protease activity has also been observed for cyclic and unnatural amino acid containing peptides (14-16). This resistance toward enzymatic digestion can be attributed to the inability of the peptide backbone to fit into the active site of the enzyme. In contrast, under the same experimental conditions KAPK was rapidly digested with $t_{1/2}$=20 min (Fig.5). A similar resistance to trypsin digestion was found for KAK.

The design of β-sheet motifs in peptides typically involves an alternating arrangement of hydrophobic and hydrophilic residues. This allows for the formation of polar and apolar surfaces on each face of the sheet (11,17,18). In such cases, hydrophobic packing of two adjacent sheets is promoted, while the hydrophilic faces provide solvent accessible sites (19,20). However, β-sheet structures are frequently seen in the interior of the hydrophobic cores of naturally occurring proteins, i.e., in areas which are not exposed to a hydrophilic environment or solvent. The β-sheet formation of KAK involves the packing of hydrophobic residues on both sides of the sheet (i.e., alanines). The lysine residues, which are concentrated at the N- and C-terminal edges, are responsible for a high aqueous solubility of the complex. In order to evaluate KAK's tertiary structure, the average molecular weight of this complex was determined by size exclusion chromatography and centrifugal concentration through ultrafiltration. Both analyses indicated a molecular weight exceeding 10^5 daltons. A β-sheet macrostructure of this size can be explained as resulting from extended hydrophobic interactions on both faces of a β-sheet, with alanine occupying both faces of the sheet. This would result in a multilayer β-sheet "dumb-bell"-like complex. Alternatively, it is conceivable that this macrostructure solubilizes itself through a micellar type complex formation, having lysine side chains at the complex/solvent interface. Further physical characterization, such as x-ray crystallography, electron microscopy or NMR, are

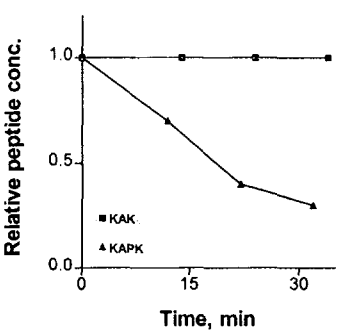

Figure 5. **Enzymatic digestion of KAK and KAPK by protease K.** 200μM of each peptide was reacted with 0.14μg/ml protease K in PBS buffer at 25°C. The relative peptide concentrations as determined by RP-HPLC are plotted as a function of the incubation time.

required to definitively establish the tertiary structure of the β-sheet character of KAK. The unusually stable nature of KAK to a wide range of perturbing conditions can be attributed to the efficient side chain packing of alanine residues. In the case of a potential micellar structure, the resulting hydrophobic core would be masked from the solvent by charged lysine residues, as is seen for some proteins having hydrophobic cores. Binding studies of KAK with a variety of biomolecules are ongoing as part of a study to evaluate the nature and role of hydrophobic cores in the binding properties of proteins. Besides representing a model for studying β-sheet formation and properties, the current studies show that it is possible to form "tertiary-like" protein structures using short peptide segments.

ACKNOWLEDGMENTS

The authors would like to thank Eileen Silva for editing the manuscript. This work was funded by National Institutes of Health grant GM 45583 and by a postdoctoral fellowship from the Spanish Ministry of Science and Education (E.P.P.).

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