Polyalanine-Based Peptides as Models for Self-Associated β-Pleated-Sheet Complexes†

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ABSTRACT: The occurrence of β-sheet motifs in a number of neurodegenerative disorders has brought about the need for the *de novo* design of soluble model β-sheet complexes. Such model complexes are expected to further the understanding of the interconversion processes that occur from cellular allowed random coil or α-helical conformation into insoluble cell-deleterious β-pleated-sheet motifs. In the present study, polyalanine-based peptides (i.e., derived from Ac-$KA_{14}K$-NH_2) were designed that underwent conformational changes from monomeric random coil conformations into soluble, macromolecular β-pleated-sheet complexes without any covalent modification. The interconversion was found to be length-, environment-, and concentration-dependent and to be driven by hydrophobic interactions between the methyl groups of the alanine side chains. A series of substitution analogs of Ac-$KA_{14}K$-NH_2 was used to study the amino acid acceptability within the hydrophobic core of the complex, as well as at both termini. The formation of amyloid plaques in a number of amyloidogenic peptides could be related to the presence of amino acids within their sequences that were found to have a high propensity to occur in these model β-sheet complexes.

The design of peptide complexes having well-defined structures appears to be increasingly feasible. Due to extensive studies using single model peptides, it now appears possible to design peptide complexes having defined secondary structures. The α-helical motif has been the most studied designed motif due to both its high frequency in naturally occurring proteins and to the relative simplicity of designing water soluble α-helical peptides with defined aggregation states. This contrasts with the inherent difficulties associated with the *de novo* design of soluble β-sheet structures. Recently, interest has arisen for the design of highly soluble, model peptide sequences that mimic naturally occurring β-sheet motifs. This is an important step in the further understanding of interconformational processes, such as those in which naturally occurring peptides undergo interconversion from cellular-allowed conformation (i.e., random coil or α-helical) to a cell-deleterious β-pleated-sheet conformation (Jarrett et al., 1993; Pike et al., 1993; Barrow & Zagorski, 1995; Nguyen et al., 1995). The development of a number of neurodegenerative disorders has been linked to conformational interconversion to highly stable, insoluble β-sheet aggregates. For example, the major component of senile plaques and cerebrovascular amyloid deposits found in the brain of patients with Alzheimer's disease is the β-amyloid protein (β-A4), an insoluble aggregated 39 to 42 amino acid fragment from the amyloid precursor protein (Wong et al., 1985; Masters et al., 1995). β-A4 fibrils are characterized by a high percentage of β-sheet structure, whereas monomeric β-A4 adopts a predominantly random coil conformation in solution (Barrow & Zagorski, 1995; Lansbury, 1995). In prion diseases, the cellular prion protein PrP^c, which is essentially α-helical (Gasset et al., 1992), is converted by a post-translational process into an abnormal self-assembling pathogenic β-sheet isoform, designated PrP^{Sc}, that accumulates in the extracellular space as amyloid plaques (DeArmond et al., 1985). A significant problem in the study of these self-assembling complexes is the extremely insoluble nature of the pathogenic β-pleated-sheet complex. To overcome this problem, efforts have been directed toward the development of simple, soluble peptide systems that permit the systematic study of interconformational changes occurring from a "native" monomeric structure to β-pleated-sheet complexes. Water solubility would also permit the development of experimental strategies to investigate the disruption of the complex and/or the inhibition of its formation.

We have recently designed a peptide that undergoes changes from a native monomeric and intramolecularly stabilized partial α-helical conformational state into a soluble, macromolecular β-pleated-sheet complex stabilized via tight intermolecular hydrophobic interactions in both faces of the resulting β-sheets (Forood et al., 1995; Pérez-Payá et al., 1996). Alanine was selected as the primary amino acid of the peptide sequence in order to obtain monomeric α-helices under mild buffer and temperature conditions; these α-helices were expected (Marqusee et al., 1989; Chakrabartty et al., 1994), and found, to be stabilized via intramolecular interactions. Hydrophilic residues (lysine in the initial series) were incorporated at both extremities and were found to provide the desired aqueous solubility. Thus, for the peptide prepared (Ac-$KA_{14}K$-NH_2), the presence of the methyl group of the alanine side chain was found to be sufficient for the induction of the required hydrophobic intermolecular interactions at high peptide concentrations. This peptide sequence was found to populate two different conformational spaces in an

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environment- and concentration-dependent manner: a partial monomeric α-helical conformation and a compact multicomponent macromolecular β-pleated-sheet structure that is extremely stable and aqueous soluble. The macromolecular complex was found to have greatly enhanced resistance to proteolytic enzymes (Forood et al., 1995; Pérez-Payá et al., 1996), as described for the pathogenic PrP^{Sc} (Oesch et al., 1985).

In the present study, the physicochemical properties of the β-pleated-sheet complexes were further investigated by circular dichroism (CD) and reversed phase high-performance liquid chromatography (RP-HPLC). This study involved 62 different peptide analogs of Ac-KA_{14}K-NH_2. Truncation and elongation analogs were prepared to study the minimum length necessary to induce the complex formation, while substitution analogs were used to determine the acceptability of amino acids on the complex formation and stability. The compactness of the complex core was characterized by determining its accessibility to intercalating agents.

MATERIALS AND METHODS

Peptide Synthesis and Purification. All peptides were prepared by simultaneous multiple peptide synthesis using *p*-methylbenzhydrylamine resin and standard Boc chemistry (Houghten, 1985; Houghten et al., 1986). Peptides were extracted with 10% acetic acid in water. To obtain the monomeric population (versus the high molecular weight complex), the peptides were treated with neat trifluoroacetic acid (TFA), followed by dilution with 9 vol. of water prior to purification. TFA is known to reduce hydrophobic interactions and has been used in other studies as a disaggregative solvent (Larsen & Holm, 1994). Preparative purification of the peptides was performed on a preparative Vydac C-18 column (Alltech Associates, Los Altos, CA) (ODS 5 μm, 25 cm × 22 mm). The peptides were stored as a lyophilized powder at −20 °C. Laser desorption time-of-flight mass spectroscopy analysis (Kratos Kompact Maldi-Tof mass spectrometer, Ramsey, NJ) was used to determine the identity of the peptides. Gentisic acid and α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) were routinely used as matrixes. Analytical RP-HPLC was carried out using Vydac C-18 columns (ODS, 3 μ, 5 cm × 4.6 mm and 5 μ, 25 cm × 4.6 mm). The mobile phase consisted of 0.05% TFA in H_2O (pH 2.5) and 0.05% TFA in acetonitrile. Peptide elution was monitored at 214 nm using a 3% per min increasing gradient. Quantitation of the RP-HPLC peak areas of the different conformation-dependent peptide populations was carried out using a Beckman System Gold software (Beckman Instruments, Fullerton, CA).

Circular Dichroism Measurements. All measurements were carried out on a Jasco J-720 CD spectropolarimeter (Eaton, MD) equipped with a Neslab RTE 110 water bath and temperature controller (Dublin, CA). CD spectra were the average of a series of three to seven scans made at 0.2 nm intervals. Peptide concentrations were determined by UV spectrophotometry at 276 nm, using the reported extinction coefficient for tyrosine [$\epsilon_{276} = 1420\ M^{-1}\ cm^{-1}$ (Marqusee et al., 1989)], or by quantitative amino acid analysis (Multiple Peptide Systems, San Diego, CA). Ellipticity is reported as mean molar residue ellipticity [θ] in degree squared centimeters per decimole. The secondary structure of peptides was estimated using the curve-fitting procedure described by Yang et al. (1986).

Fourier Transform Infrared Spectroscopy. The spectra were recorded on a Impact 400 FTIR spectrophotometer (Nicolet Analytical Instrument, Madison, WI) at 2 cm^{-1} resolution using the KBr disc method. One thousand scans were averaged for data recorded from 400 to 4000 cm^{-1}. The aggregated form was obtained following an overnight incubation at 65 °C of a 5 mg/mL solution of monomeric Ac-KYA_{13}K-NH_2 and lyophilization. KBr discs were prepared using a peptide:KBr weight ratio of 1:100.

Microscopic Measurements. The aggregated form of Ac-KYA_{13}K-NH_2 was obtained following an overnight incubation at 60 °C of a 20 mg/mL solution of the monomeric form. Of the aggregate suspension, 10 μL was placed on a microscope slide, air-dried at 50 °C, rinsed with water, and stained with saturated solution of congo red in 80% ethanol. After successive washes with water, 80% ethanol, absolute ethanol, and water, the stained sample was visualized by bright field and polarized light microscope (magnification x400).

Negatively stained fibrils were prepared by spotting 5 μL of the aggregated suspension onto carbon-coated copper grids. The samples were stained with 2% (w/v) uranyl acetate in water. The air-dried sample was visualized by electron microscopy at 100 kV (Philips EM-300).

Reaction of DTNB with Cysteine-Containing Peptides. Stock solutions of the monomeric or aggregated forms of Ac-KYA_7CA_5K-NH_2 and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma, St. Louis, MO) were prepared in 50 mM Tris (pH 7.4). Appropriate volumes of each stock solution were mixed to reach a final concentration of peptide and DTNB of 150 and 750 μM, respectively. The appearance of 2-nitro-5-thiobenzoate anion was followed spectroscopically by monitoring the increase in optical density (OD) at 410 nm (Dekin et al. 1963) using a Hewlett-Packard 8452A dioder spectrophotometer (Fullerton, CA). RP-HPLC and mass spectrometry were used to assess the completeness of the peptide's chemical modification.

RESULTS

Characterization of Polyalanine−Based Peptides. The synthesis of the peptide Ac-KA_{14}K-NH_2 was shown in a previous study to result in the formation of two distinct entities (Forood et al., 1995). The main population eluted as a broad peak in RP-HPLC and was found to be a macromolecular complex (molecular mass exceeding 10^5 Da) having a clear β-sheet conformation. The residual population (less than 10% of the original crude peptide) eluted earlier as a sharp peak during RP-HPLC, which was found to be a monomeric peptide showing a partial α-helical CD spectrum [53% α-helical conformation as estimated using Yang et al. (1986) algorithm (Figure 1)]. Furthermore, the θ_{222}:θ_{208} ratio was indicative of the presence of a single-stranded α-helical conformation [ratio lower than 1 (Zhou et al., 1992)]. The substitution by a tyrosine residue at the N-terminal region (Ac-KYA_{13}K-NH_2) did not modify the conformational behavior of the model peptide, and enabled a more accurate determination of the peptide concentration (Pérez-Payá et al., 1996). It should be noted that the spectrum observed for the β-sheet complex does not precisely match the CD spectra expected for such a conformation. In a standard

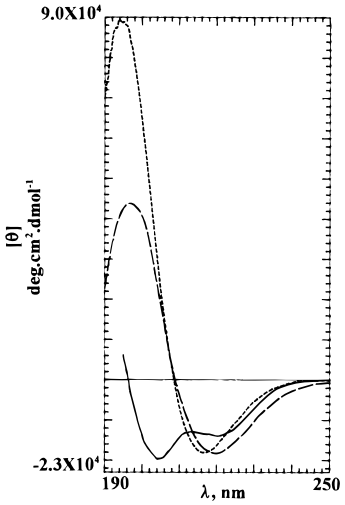

FIGURE 1: CD spectra of monomeric and complexed Ac-KYA$_{13}$K-NH$_2$. The CD spectra were recorded in 5 mM MOPS buffer, pH 7, at 25 °C. (—) Monomeric state at 100 μM; (···) complexed form obtained after overnight incubation at 65 °C of a 1.8 mM solution and posterior dilution to 100 μM; (− −) Ac-LKLKLKLKLK-LKLKLK-NH$_2$ in the presence of 2.47 mM SDS (Blondelle et al. 1995a) as a standard β-sheet conformation control.

β-sheet conformation, the absolute intensity of the positive band (maximum at 192−197 nm) should be twice the intensity of the negative band (minimum at 216−218 nm). This is depicted in Figure 1 for an amphipathic leucine−lysine-based peptide that was used here as an internal reference (Blondelle et al., 1995a). In contrast, the CD spectrum of the macromolecular β-sheet complex was found to have a positive band 5 times larger in absolute intensity than the negative band (Figure 1).

The conversion to β-sheet conformation was confirmed by FTIR (Figure 2). Following an overnight incubation at 65 °C of a 5 mg/mL solution of the monomeric form of Ac-KYA$_{13}$K-NH$_2$, the FTIR spectra showed a predominant β-sheet conformation with an amide I band at 1630 cm^{-1} (86% β-sheet conformation as determined by RP-HPLC). In contrast, the starting monomeric form of Ac-KYA$_{13}$K-NH$_2$ showed a FTIR spectra of a random coil or α-helical conformation with an amide I band at 1657cm^{-1} (Figure 2).

Due to the apparent irreversibility of the monomeric form to macromolecular β-sheet interconversion, the pure monomeric populations of Ac-KYA$_{13}$K-NH$_2$ and its analogs were used as starting materials in the present studies. Such populations were found to be readily obtained following TFA treatment and successive purification of the crude peptides (see Materials and Methods).

Concentration and Temperature Dependence of the Peptides' Self-Aggregation. The occurrence and rate of the interconversion to a macromolecular β-sheet was found in earlier studies to be environment and concentration-dependent. In particular, preliminary studies showed that complete conversion of the monomer to the macromolecular complex could be achieved following overnight incubation at 65 °C. We have further evaluated the experimental conditions that result in complete interconversion by monitoring the two populations by RP-HPLC. The concentration dependence of this process was first analyzed by incubating at 65 °C varying concentrations of Ac-KYA$_{13}$K-NH$_2$ in 5mM MOPS, pH 7.0 (Figure 3). At low peptide concentration (concentration lower than 0.5 mM), no significant change in the RP-HPLC profile was observed even after 200 h incubation. The CD spectra of the peptide prior to and following the incubation showed similar partial α-helical conformations (data not shown). Increased concentrations of Ac-KYA$_{13}$K-NH$_2$ progressively resulted in the occurrence of the interconformational process to the β-sheet conformation. As shown in Figure 3, the β-sheet complex formation was found to be independent of the incubation time. The profile of the concentration-dependence curve observed for the interconversion indicates that Ac-KYA$_{13}$K-NH$_2$ may form micelle-like aggregates (Figure 3) with a critical concentration of 1 mM at 65 °C.

The effect of temperature on the formation of the observed Ac-KYA$_{13}$K-NH$_2$ β-pleated-sheet complex was investigated in order to determine if the self-assembly process was stabilized by the "hydrophobic effect" or by a competition between inter- and intramolecular hydrogen bonds. As shown in Figure 4, the β-pleated-sheet complex formation was found to be significantly increased upon temperature elevation. These results support the premise that the stabilization is resulting from hydrophobic interactions and, in turn, in the formation of a micellar type of aggregate. Under the conditions studied (3 h incubation, peptide concentration of 1.8 mM), the critical temperature for complex formation was estimated at 45 °C.

Effect of the Length of the Alanine Motif and Terminal Charges upon the β-Pleated-Sheet Complex Formation. If one begins with the premise that the hydrophobic section of Ac-KYA$_{13}$K-NH$_2$ is responsible for the observed β-pleated-sheet complexing properties, then these properties are expected to be modulated by the length of this motif. In order to study these premises, a series of analogs varying in length from 3 to 25 consecutive alanine residues were prepared (i.e., Ac-KYA$_n$K-NH$_2$, $n = 3-25$). Following their syntheses, the peptides were converted to their monomeric form by the TFA treatment described above and purified. The complex formation was quantitatively monitored by RP-HPLC and qualitatively assessed by CD spectroscopy. These peptides could be separated into three distinct groups (Figure 5). The first group included those peptides that were not interconverted into the β-sheet complex. These were characterized by $n \leq 9$. The second group was made up of peptides with $10 \leq n \leq 14$, which showed varying levels of complex formation. Their CD analysis revealed the coexistence of the β-sheet and the partial α-helical structures (results not shown). Finally, the third group was made up of those peptides having $n \geq 15$, for which the interconversion process from the monomeric to the β-sheet complex was complete.

The ability of Ac-KYA$_n$K-NH$_2$ to form soluble β-pleated-sheet complexes involves the packing of hydrophobic residues on both sides of the sheet, while the lysine residues, which are concentrated at the N- and C-terminal edges, are responsible for the observed high aqueous solubility (>10 mg/mL). It is conceivable that the solubility of this macrostructure is due to the arrangement of the lysine side chains at the complex/solvent interface. The effect of this anticipated charge density at the interface was studied using a set of substitution analogs of Ac-KYA$_{13}$K-NH$_2$. The presence of negative charges (Ac-EA$_{13}$E-NH$_2$), as well as of ion pairs (zwiterionic analog Ac-KEA$_{13}$KE-NH$_2$), resulted in complex formation. Similarly, substitution of lysine residues by histidines (Ac-HYA$_{13}$H-NH$_2$) or the presence of

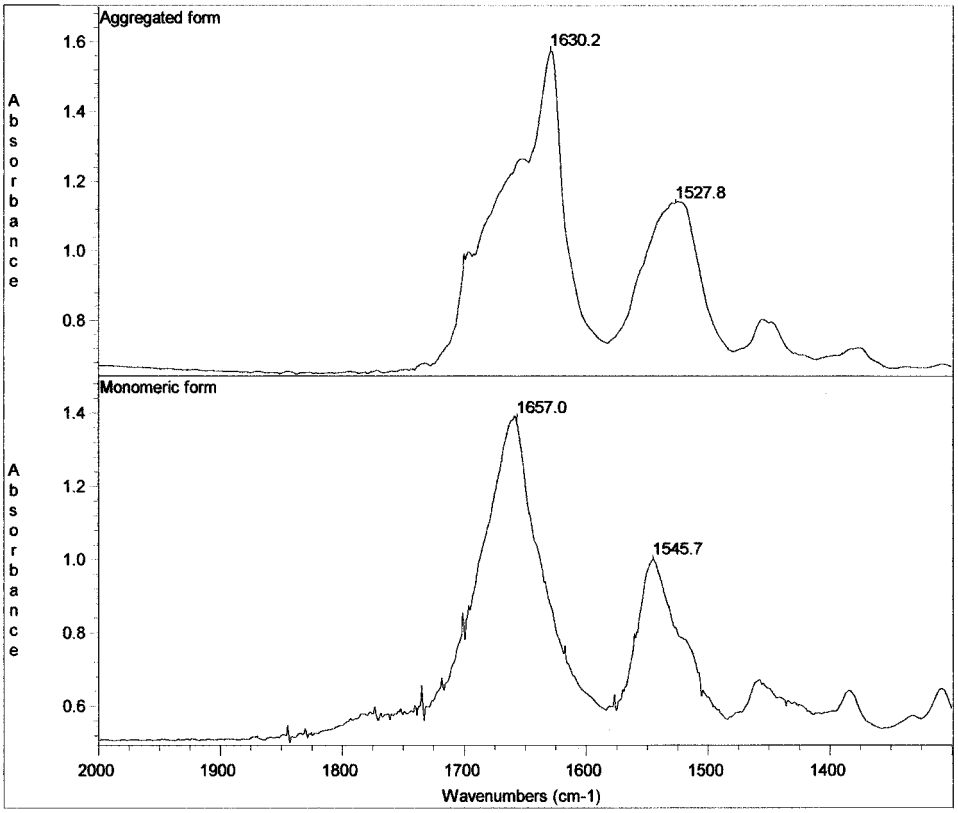

FIGURE 2: Solid-state (KBr) FTIR spectra of Ac-KYA$_{13}$K-NH$_2$. Top spectra: following overnight incubation at 65 °C. Bottom spectra: monomeric form prior to overnight incubation.

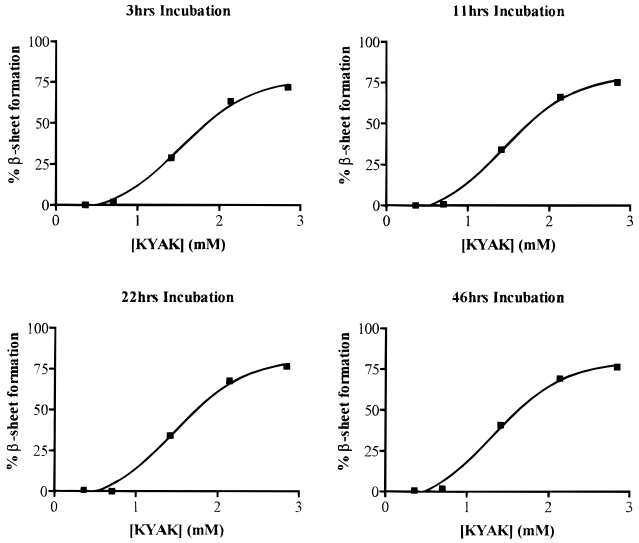

FIGURE 3: Dependence of % β-pleated-sheet complex formation on peptide concentration. The percentage of complex formation by Ac-KYA$_{13}$K-NH$_2$ was quantitatively monitored by RP-HPLC (see Materials and Methods) after 3, 11, 22, or 46 h incubation at 65 °C using different peptide concentrations.

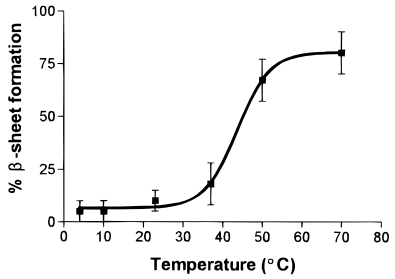

FIGURE 4: Dependence of % β-pleated-sheet complex formation on incubation temperature. The percentage of complex formation by Ac-KYA$_{13}$K-NH$_2$ was quantitatively monitored by RP-HPLC (see Materials and Methods) at temperatures varying from 5 to 65 °C and a constant peptide concentration of 1.8 mM.

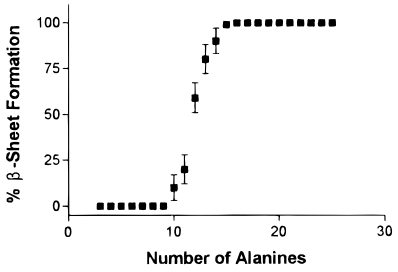

FIGURE 5: Dependence of % β-pleated-sheet complex formation on peptide length. The percentage of complex formation by Ac-KYA$_n$K-NH$_2$ peptides at 1.4 mM was quantitatively monitored by RP-HPLC (see Materials and Methods) after 24 h incubation at 65 °C.

a free α-amino group (KYA$_{13}$K-NH$_2$) did not disrupt the ability of these peptides to form β-sheet complexes. In contrast, the presence of two consecutive positively charged side chains (Ac-KKYA$_{13}$KK-NH$_2$) eliminated the complex formation. These results suggest that intermolecular hydrophobic interactions are not strong enough in these cases to compensate for the energetically unfavorable positioning of two consecutive lysine side chains.

Amino Acid Acceptability within the Hydrophobic Core. The insertion of a single proline residue in the middle of the alanine stretch was found earlier to result in a peptide lacking the β-sheet population (Forood et al., 1995). The CD characterization of this analog (Ac-KA$_5$PA$_8$K-NH$_2$) showed a predominantly random coil conformation (Forood et al., 1995). The effect of proline on the ability of the polyalanine-based peptides to form the β-pleated-sheet

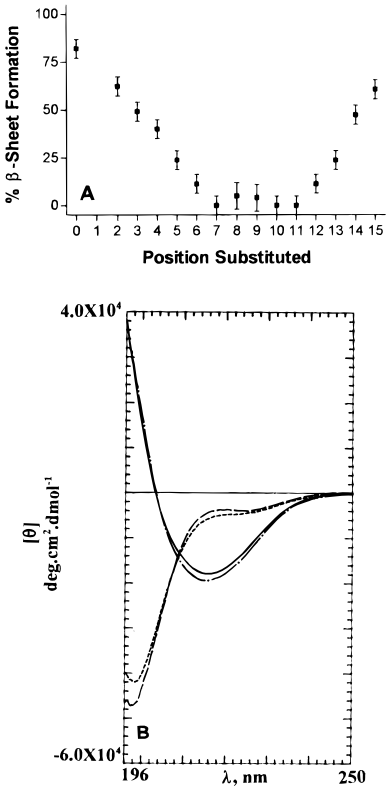

FIGURE 6: Dependence of % β-pleated-sheet complex formation for the proline substitution analogs of Ac-KA_{14}K-NH_2. (A) The percentage of complex formation was determined as described in Figure 5. (B) CD spectra of representative peptides recorded in 5 mM MOPS buffer, pH 7, at 25 °C at a peptide concentration of 100 μM, (—) Ac-KPA_{13}K-NH_2, (– –) Ac-KA_5PA_8K-NH_2, (•••) Ac-K$A_{10}PA_3$K-NH_2, and (—•—) Ac-KA_{13}PK-NH_2.

complex was further investigated using a complete set of 14 single proline substitution analogs of Ac-KA_{14}K-NH_2. "Walking" a proline from the N- to C-terminus in the Ac-KA_{14}K-NH_2 sequence resulted in a "valley" profile of β-sheet peptide formation as determined by RP-HPLC and CD (Figure 6, panels A and B). A gradual decrease in β-sheet content was observed upon substituting alanine from position 2 to position 7, where no β-sheet structure was detected. Those analogs in which alanine-7 to alanine-11 were replaced by a proline adopted a random coil conformation. A steep increase in the percentage of β-sheet formation was then observed as the proline was moved toward the C-terminus. A similar valley effect was seen in an earlier RP-HPLC study upon walking proline through the sequences of monomeric polyalanine peptides (Büttner et al., 1992).

While proline is one of the most destabilizing secondary structure amino acids (Chou & Fasman, 1974; Richardson & Richardson, 1988; O'Neil & DeGrado, 1990; Chakrabartty et al., 1994), the question that arises from the above results is the acceptability of other amino acids inside the hydrophobic core of the β-sheet complex. Similar RP-HPLC and CD studies were carried out using single substitution analogs of Ac-KYA_{13}K-NH_2 at the position where proline exerted its greater destabilizing effect (i.e., substituting alanine-10). A new series of 20 peptides (Ac-KYA_7OA_5K-NH_2; O represents one of the 20 L-amino acids) was synthesized, treated with TFA, and purified as described above. The peptides' ability to self-aggregate in a macromolecular β-sheet complex was initially evaluated under the experimental conditions found for Ac-KYA_{13}K-NH_2 to result in

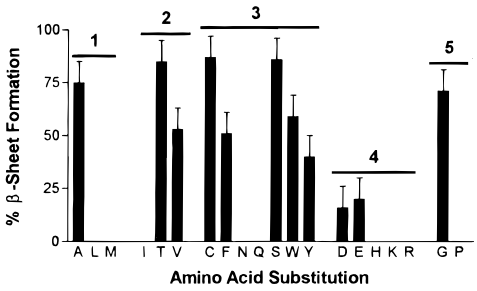

FIGURE 7: Effect of amino acid substitutions in Ac-KYA_7OA_5K-NH_2 on the % β-pleated-sheet complex formation. The percentage of complex formation was determined as described in Figure 4. The amino acids are grouped as (1) aliphatic non-β-branched, (2) aliphatic β-branched, (3) noncharged β-substituted, (4) charged, (5) secondary structure destabilizers.

complete complex formation (1.4 mM peptide concentration, 48 h incubation at 65 °C; Figure 7). Interestingly, no complex formation was detected when an aliphatic non-β-branched amino acid other than alanine was at the guest position. In contrast, Ac-KYA_7LA_5K-NH_2 and Ac-KYA_7MA_5K-NH_2 adopted a partial α-helical structure as shown by CD spectroscopy (results not shown). In the case of aliphatic β-branched amino acids, threonine was found to stabilize the complex in a manner similar to alanine, while lower β-sheet content was obtained when valine was at position 10. Furthermore, a complete inhibition of the complex formation was observed when isoleucine was located at this position. When the guest position was held by a noncharged β-substituted amino acid, the rank order of allowance was found to be Cys $\approx$ Ser > Phe > Tyr $\approx$ Trp $\gg$ Gln, Asn. While the presence of a tryptophan or a tyrosine residue led to the same amount of macromolecular complex as determined by RP-HPLC, a mixture of α and β conformation was observed for Ac-KYA_7WA_5K-NH_2. These two peptides were, however, found to adopt a macromolecular conformation of similar molecular weight as determined by size exclusion chromatography. Little (<20%) or no complex formation was detected upon inserting a charged amino acid within the polyalanine sequence. Finally, in contrast to proline, glycine, which is considered as a secondary structure destabilizing residue (Chou & Fasman, 1974), allowed the β-pleated-sheet complex formation.

Overall, these results show that the substitution of a single alanine can dramatically affect the formation of β-pleated-sheet complexes (0−90% β-sheet content was observed according to the substituting amino acid). As anticipated, the critical micellar concentration and temperature for the different analogs were found in preliminary studies to differ from one analog to the other. For example, >85% β-sheet complex formation was observed for the serine substitution analog Ac-KYA_7SA_5K-NH_2 at concentration as low as 0.35 mM at 65 °C and at temperature as low as 25 °C at 3 mM.

Evaluation of the Compactness and Stability of the Hydrophobic Core. Initial studies using size exclusion chromatography and centrifugal concentration through ultrafiltration indicated that the polyalanine β-sheet complex has a tertiary structure of an average molecular mass exceeding 10^5 Da (Pérez-Payá et al., 1996). Moreover, the occurrence of a CD signal in the near UV region indicated the occurrence of a compact tertiary structure (Pérez-Payá et al., 1996). In order to further study the compactness and stability of the tertiary structure of the β-pleated-sheet

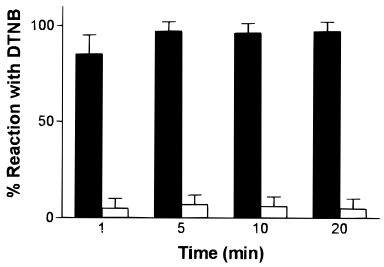

FIGURE 8: Accessibility of the cysteine residue in the monomeric and complexed form of Ac-KYA_7CA_5K-NH_2. The accessibility of the cysteine residue in Ac-KYA_7CA_5K-NH_2 was monitored by mixed disulfide formation with DTNB as a function of reaction time for the monomeric form (black bars) and for the complexed form (hollow bars) of Ac-KYA_7CA_5K-NH_2.

complex, we have examined the physical accessibility of small molecules within the complex core using the peptide analog Ac-KYA_7CA_5K-NH_2. At low peptide concentration, Ac-KYA_7CA_5K-NH_2 exists as a monomer, while the complexed form was obtained at concentrations higher than 2 mM and after incubation at 65 °C for 48 h. Dilutions of this solution did not dissociate the complex. The accessibility of the cysteine residue was monitored by determining the formation of mixed disulfide with DTNB. As shown in Figure 8, the cysteine residue in the monomeric form is fully available to react with DTNB, suggesting a solvent accessible position for this residue. In contrast, very low ratios of mixed peptide-DTNB disulfide were obtained for the complexed form of Ac-KYA_7CA_5K-NH_2. This result suggests that the tertiary structure of the β-pleated-sheet is highly compact and that small molecules cannot be used to disrupt the complex by intercalation in the core.

Fibrils Formation. The ability of the Ac-$KYA_{13}K$-NH_2 complex to form fibrils in a manner similar to amyloidogenic peptides was evaluated by congo red staining methods and electron microscopy. The aggregated form of Ac-$KYA_{13}K$-NH_2 was found to bind to congo red by bright light optical microscopy resulting in a yellow-green birefringent under polarized light somewhat different from the green birefringent reported for amyloidogenic peptides (data not shown). This may be due to a different orientation of the congo red molecule while binding to Ac-$KYA_{13}K$-NH_2 as compared to amyloidogenic peptides. On the other hand, the formation of fibrils, although of smaller size than those reported for amyloidogenicpeptides (Merz et al., 1983), was clearly seen by electron microscopy (Figure 9).

DISCUSSION

Polyalanine-based peptides were found to underscore conformational conversions leading to the formation of β-pleated-sheet complexes similar to those occurring in neurodegenerative diseases. For example, similarity to β-amyloid-like structures could be seen by CD spectroscopy. Thus, the CD spectra observed for aggregated Ac-$KYA_{13}K$-NH_2 was similar to the spectrum reported for the structure of human amylin in the presence of 6.25% 1,1,1,3,3,3-hexafluoro-2-propanol (Cort et al., 1994). This was felt to be due to the stacking of individual β-sheets and/or sheet distortion upon the stacking arrangement. Furthermore, it is noteworthy that the aggregated form of Ac-$KYA_{13}K$-NH_2 was found earlier to bind to fluorescent probes at concentrations above its micellar critical concentration, reflecting the presence of a hydrophobic core that excludes water upon complex assembly (Pérez-Payá et al., 1996). Similar surfactant properties were recently reported for the β-amyloid protein based on its ability to lower the surface tension of water and to bind to fluorescent probes (Soreghan et al., 1994). Due to the high aqueous solubility of the polyalanine-based β-pleated-sheet complexes, these peptides represent good model systems for studying such interconformational conversion processes. Not only does the solubility simplify the study of experimental conditions involved in the interconformational process, but the simplicity of these model peptide systems also relies on the fact that the interconversion process occurs without any covalent modification. Furthermore, the fact that high temperatures enhance the self-aggregation of the polyalanine peptides indicates that a hydrophobically driven process predominates (O'Shea et al., 1989).

Since alanine shows one of the highest helical propensities in all of the reported helicity scales (Chou & Fasman, 1974; Richardson & Richardson, 1988; O'Neil & DeGrado, 1990; Chakrabartty et al., 1994), the conversion into a macromolecular β-sheet complex found for Ac-$KYA_{13}K$-NH_2 and various analogs is of note. β-sheet structures have been observed during the solid phase synthesis of polyalanine peptides (Hendrix et al., 1990; Larsen & Holm, 1994). Poly(ethylene glycol) bound homo-oligoalanines were also reported to adopt β-sheet or random coil conformations in aqueous solution (Toniolo et al., 1979). Similar formation of macromolecular β-sheet complexes has recently been reported for Ac-$D_2Q_{15}K_2$-NH_2 (Perutz et al., 1994). Glutamine repeats have been found in natural proteins that are linked to several inherited neurodegenerative diseases. Interestingly, as with alanine, the β-sheet forming tendency of glutamine was found to be low when measured independently in two separate model systems (Minor & Kim, 1994a; Smith et al., 1994). Whether or not other noncharged residues also have potential to form such complexes remains to be determined.

In contrast, short alanine-based peptides (e.g., derived from the sequence Ac-$A_4KA_4KA_4KA$-NH_2) were reported to form monomeric α-helices in aqueous solution (Marqusee et al., 1989; Chakrabartty et al., 1994). The lack of complex formation found in the present study for the substitution analog Ac-KYA_7KA_5K-NH_2, which was found to adopt a partial α-helical conformation, correlates with these earlier findings. If one envisions such peptides in a β-sheet structure, then the presence of lysine residues in the middle of alanine sheets would result in electrostatic repulsion. This, in turn, would prevent intermolecular interactions necessary for β-structure stabilization. Interestingly, it has recently been demonstrated that short alanine-based peptides may exhibit highly amyloidogenic properties (Gasset et al., 1992). Although these peptides were predicted to correspond to four α-helical regions of the prion protein sequences, three out of four of these synthesized peptides formed amyloids. The most highly amyloidogenic peptide found was AGAAAAGA (which corresponds to Syrian hamster PrP residues 113−120). The insertion of a glycine into polyalanine stretches was also found in the present study to lead to the formation of β-pleated-sheet complexes. In unrelated studies, repeat units of alanine/glycine have been reported to occur in β-sheet structures in polypeptides deriving from spiders' native silk proteins (Prince et al., 1995).

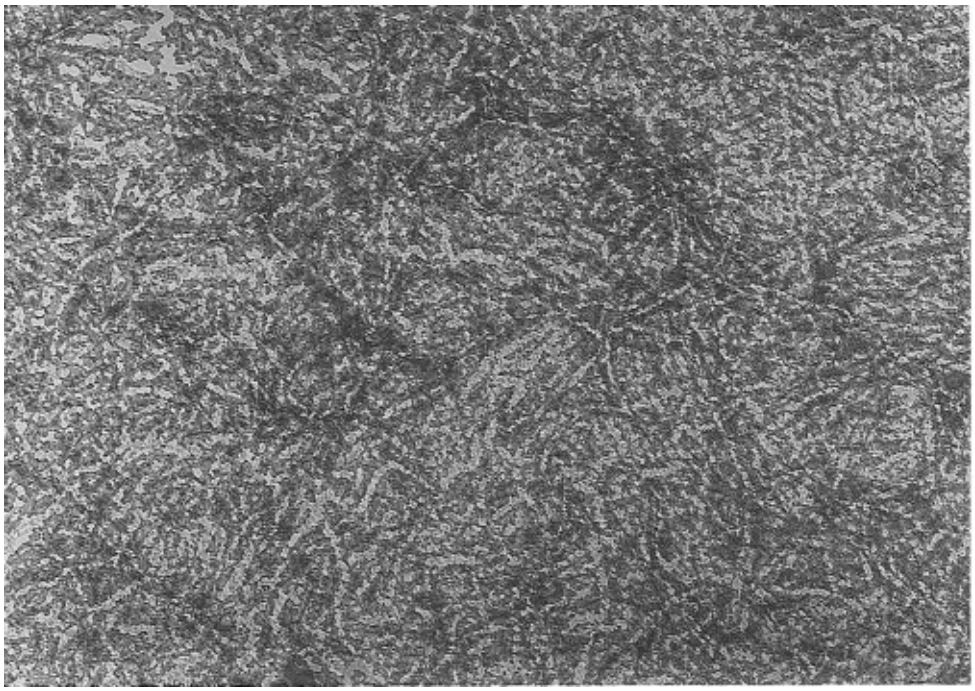

FIGURE 9: Electron micrograph of negative-stained preparation of fibrils of aggregated Ac-KYA$_{13}$K-NH$_2$. The peptide was stained as described in the Materials and Methods with 2% uranyl acetate. The sample was viewed with a Phillips electron microscope. Magnification, $\times$ 73000.

Table 1: Analysis of Amyloidogenic Peptide Sequences in Terms of βps Amino Acids[a]

sequence	peptide[b]	fibril formation[c]	% β-sheet formation[d]	% of residues with tendency to form β-pleated-sheet
Ac-MKHMAGAAAAGAVV-NH$_2$	H1	+	60	71
Ac-MLGSAMSRPMMHF-NH$_2$	H2	−	23	38
Ac-CDVNITIKQHTVTT-NH$_2$	H3	+	55	57
Ac-DIKIMERVVEQMCTTQY-NH$_2$	H4	+	70	47
AGAAAAGA	SHaPrP-(113−120)	+	80	100
AGAAAAGAVVGGLGG	SHaPrP-(113−127)	+	70	93
Ac-VVGGLGGYMLGSAMSR	SHaPrP-(121−136)	−	14	69
DAEFRHKSGYEVKKQKLVFFAEDVGSNKGAIIGLMVGGVVIAT	βA4-(1−43)	+[e]	nr[h]	65
RSFFSFLGEAFKGAR	AA15	+[f]	nr	80
KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH$_2$	hAM	+[g]	β-cd	62
KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY-NH$_2$	rAM	−[g]	α-cd	51

[a] βps amino acids are defined as those amino acids which led to complex formation when at position 8 in Ac-KYA$_7$OA$_5$K-NH$_2$ (see Results). [b] H1−H4 represent the four helices of the mammalian PrP; ShaPrP = Syrian hamster PrP; βA4 = β-amyloid; AA15 = sequence 1−15 of human AA amyloid; hAM = human amylin; rAM = rat amylin. [c] As determined by Fourier transform IR spectroscopy (Gasset et al. 1992). [d] Determined by electron microscopy (Gasset et al. 1992). [e] Reported in ref (Merz et al. 1983). [f] Reported in ref (Soto et al. 1995). [g] Reported in ref (Cort et al. 1994). [h] nr: not reported. α-cd: Coil-helix transition upon HFIP titration as determined by CD spectroscopy (Cort et al. 1994). β-cd: β-sheet in 6.25% HFIP as determined by CD spectroscopy (Cort et al. 1994).

The substitution of a single alanine residue in Ac-KYA$_{13}$K-NH$_2$ by one of the 20 L-amino acids led to a wide range of β-sheet complex formation (0−90%). For example, the β-sheet disruptive effect of a proline residue can be explained by the rigidity of the proline ring, which prevents the occurrence of sheet stacking. Similarly, the lack of β-sheet complex formation observed upon substituting alanine-10 with methionine, leucine, or isoleucine, may be due to the bulkier side chains of these amino acids relative to alanine, which could be expected to prevent the efficient packing of the multilayered sheets and, in turn, to favor monomeric structures. Finally, electrostatic repulsion between two sheets can explain the lack of β-sheet complex formation for those analogs containing a charge amino acid at position 10. The β-pleated-sheet complex forming tendencies of each amino acid derived from these studies do not show any correlation with statistical (Chou & Fasman, 1974) or experimentally (Minor & Kim, 1994a; Smith et al., 1994) determined β-sheet propensities. These findings indicate that secondary structure propensity values cannot solely account for conformational preferences of a given amino acid, but that other factors such as the hydrophobic effect and side chain steric allowance must also be taken into consideration. These results agrees with the context dependence of β-sheet propensities suggested by the lack of correlation observed between the propensities of amino acids when located at the edge or at the center of the same protein G(GB1) (Minor & Kim, 1994b).

In order to further analyze the amyloid formation at the amino acid sequence level, we have compared the sequences of known amyloidogenic peptides in terms of their residue propensity to occur in a β-pleated-sheet complex (Table 1). Good correlation was seen between the formation of amyloid plaques and the presence of clusters of amino acids found

in the present study to have a high propensity to occur in β-pleated-sheet complexes (βps amino acids). For instance, while only 47% of βps amino acids are present in the amyloidogenic H4 peptide, these amino acids are distributed in the cluster valine-8 to tyrosine-17. Similarly, the Alzheimer peptide βA4-(1−43) contains 65% βps amino acids distributed in two clusters: valine-18 to serine-26 and valine-36 to threonine-43 (100 and 88% of βps, respectively). On the other hand, peptide SHaPrP-(121−136), which contains 69% βps amino acids, did not form amyloid fibrils. This may be due to the presence of non-β-pleated-sheet forming amino acids inserted between every three or four βps amino acids.

In conclusion, the present studies confirm earlier findings that polyalanine-based peptides represent useful model systems to study interconformational processes, as they may relate to similar processes occurring in neurodegenerative diseases. Due to their excellent aqueous solubility that permits the straightforward monitoring of such interconformational processes, these peptides may be used to develop new strategies for the inhibition of the formation of deleterious amyloid plaques. Among those strategies, the use of nonsupport-bound synthetic combinatorial library techniques [reviewed in Blondelle et al. (1995b)] to identify inhibitory peptides or peptidomimetics of β-pleated-sheet complexes are currently being investigated.

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