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A peptide study of the relationship between the collagen triple-helix and amyloid

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Abstract

Type XXV collagen, or Collagen-Like Amyloidogenic Component (CLAC), is a component of amyloid plaques, and recent studies suggest this collagen affects amyloid fibril elongation and has a genetic association with Alzheimer's disease. The relationship between the collagen triple helix and amyloid fibrils was investigated by studying peptide models, including a very stable triple helical peptide (Pro-Hyp-Gly)₁₀; an amyloidogenic peptide GNNQQNY; and a hybrid peptide where the GNNQQNY sequence was incorporated between (GPO)_n domains. CD and NMR spectroscopy showed the GNNQQNY peptide formed a random coil structure, while the hybrid peptide contained a central disordered GNNQQNY region transitioning to triple-helical ends. Light scattering confirmed the GNNQQNY peptide had a high propensity to form amyloid fibrils, while amyloidogenesis was delayed in the hybrid peptide. NMR data suggested the triple-helix constraints on the GNNQQNY sequence within the hybrid peptide may disfavor the conformational change necessary for aggregation. Independent addition of a triple-helical peptide to the GNNQQNY peptide under aggregating conditions delayed nucleation and amyloid fibril growth. The inhibition of amyloid nucleation depended on the Gly-Xaa-Yaa sequence and required the triple-helix conformation. The inhibitory effect of the collagen triple-helix on an amyloidogenic sequence, when in the same molecule or when added separately, suggests type XXV collagen, and possibly other collagens, may play a role in regulating amyloid fibril formation.

Keywords

Collagen; Triple helix; Light Scattering; CD; NMR; amyloid; aggregation

Introduction

A number of recent studies suggest a relationship between the extracellular matrix protein collagen and the formation of amyloid fibrils ¹⁻³. Amyloid plaques are found to contain a collagen, denoted as Collagen-Like Amyloidogenic Component (CLAC) or type XXV collagen, and this soluble protein was derived from a transmembrane protein. Alleles of the type XXV collagen gene (COL25A1) were found to be associated with increased risk for Alzheimer's disease (AD) in a Swedish population ⁴. In transgenic mice, overexpression of COL25A1 led to an AD-like phenotype as a result of an increase in the β -site APP-cleaving enzyme 1, intracellular aggregation and extracellular matrix deposits of A β protofibrils⁵. *In*

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vitro studies have suggested that type XXV collagen binds to aggregated A β and impedes the formation and elongation of amyloid fibrils². Type XXV collagen also confers an increased protease resistance to amyloid fibrils⁶. Other links between collagen and amyloid include the ability of type VI collagen to protect against A β neurotoxicity in neurons¹ and a possible role for collagen in promoting amyloid formation of β_2 -microglobulin during kidney dialysis³. Because of these reports, the interaction between the collagen triple-helix motif and amyloid merits further investigation.

Collagen and amyloid have distinct molecular structures and amino acid sequence features. The amyloid structure can be generated from a wide range of native or disordered proteins⁷, and is also formed by peptides, such as the amyloid-forming peptide identified from the yeast prion Sup35⁸. The defining feature of collagen is the triple-helical structure, where three polypyrrolone II-like chains are supercoiled around a common axis. The close packing of the three chains requires every third residue to be Gly, generating the typical (Gly-Xaa-Yaa)_n repeating sequence. In addition, a high content of the imino acids Pro and hydroxyproline (Hyp) stabilize the extended polypyrrolone II-like structure. Although the repeating tripeptide pattern is perfect in fibrillar collagens, there are interruptions in this sequence in all non-fibrillar collagens⁹⁻¹¹ and such non Gly-Xaa-Yaa sequences may have implications for collagen-amyloid interactions. For instance, type XXV collagen has N- and C-terminal non-collagenous domains flanking a (Gly-Xaa-Yaa)_n collagenous domain which is interrupted four times, by two small 4-residue imperfections and two large non-collagenous sequences, 24 and 26 residues in length (denoted as NC2 and NC3, respectively)¹². Part of the NC2 non-collagenous domain, which is flanked by triple-helix domains, was found to be essential for the ability of type XXV collagen to delay amyloid fibril formation¹². In addition, recent studies indicate that some of the interruptions between (Gly-Xaa-Yaa)_n domains in non-fibrillar collagens have amyloidogenic potential and one 9 residue interruption sequence from type IV basement membrane collagen was shown to form amyloid-like fibrils with a cross- β structure which binds to Thioflavin T¹³.

Peptide models have been useful for defining the conformation, stability and aggregation of both triple-helices and amyloid. The high resolution structures of (Pro-Hyp-Gly)₁₀, denoted as (POG)₁₀,¹⁴ and almost 20 other collagen model peptides have been obtained¹⁵, and a host-guest peptide set has been used to quantitate triple-helix propensity of different Gly-Xaa-Yaa triplets¹⁶. In addition, the self-association of (POG)₁₀ and a number of triple-helical peptides, have been characterized, to understand the factors promoting self-association of triple-helices^{17,18}. For Sup35 the residues responsible for aggregation have been defined⁸ and recently, the molecular structural features of a small peptide with the minimum amyloidogenic sequence from Sup35, GNNQQNY has been determined by x-ray diffraction^{8,19-21} and solid-state NMR²²⁻²⁶. The structure of this seven-residue fragment revealed the presence of the typical “cross-beta” motif and a steric zipper (pair of β -sheets mated closely together by intermeshing side chains) that has been suggested to be the basis of amyloid fibrils in general. The presence of good model peptide systems for both the collagen triple-helix and amyloid systems provides an opportunity to study basic features of the interaction between these two motifs, and how the triple-helix affects amyloid aggregation.

This study focuses on the triple-helical peptide (POG)₁₀ and the amyloidogenic GNNQQNY peptide. A hybrid peptide was designed where the GNNQQNY sequence is incorporated between (GPO)_n domains, (GPO)₅GNNQQNY(GPO)₄. This serves as a model for non-fibrillar collagens which contain amyloidogenic domains flanked by triple-helices¹³. The constraints of GNNQQNY attachment to triple-helices on both sides within the hybrid peptide was found to lead to delayed aggregation. To model the effect of type XXV and other collagens on amyloid fibril formation, collagen model peptides were added to the

GNNQQNY peptide under associating conditions. The peptide (POG)₁₀ increased the lag phase and slowed the formation of aggregates, and comparison with other collagen model peptides indicated the triple-helix conformation is required for the increased lag phase and that a positively charged triple-helix is less inhibitory.

Materials and Methods

Peptides

The peptide (POG)₁₀ was obtained from Peptide International, Louisville, KY. The GNNQQNY and the hybrid peptides were synthesized by Tufts University Core Facility, Boston, MA. A peptide of sequence (GPO)₂GROGKRGKO(GPO)₃GG was synthesized as previously described²⁷. A peptide with a sequence similar to (POG)₁₀, but missing one Gly, (POG)₄PO(POG)₅, was synthesized previously and shown to be unable to form the triple helical structure²⁸. Peptides were purified on a Shimadzu reverse-phased high pressure liquid chromatography (HPLC) systems and purity was confirmed by mass spectrometry using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF). From HPLC and mass spectrometry, the purity of the GNNQQNY peptide is estimated to be 95%. GNNQQNY and hybrid peptides are blocked with acetylation of the N-terminus and amidation of the C-terminus. Concentrations of (POG)₁₀ peptide solutions were estimated by obtaining the absorbance at 214 nm using $\epsilon_{214} = 2200 \text{ M}^{-1}\text{cm}^{-1}$. For the peptides containing a Tyr residue, concentration was determined at 275nm using $\epsilon_{275} = 1400 \text{ M}^{-1}\text{cm}^{-1}$.

All studies were carried out in 20mM sodium phosphate, 150mM NaCl, pH=6. A value of pH=6 was selected instead of pH=7 because it allows amide proton information in the spectra by reducing the hydrogen exchange rate for NMR experiments.

Circular Dichroism (CD) spectroscopy

CD measurements were conducted using the Aviv model 62DS spectrophotometer equipped with a Peltier temperature controller. Prior to measurements, peptide solutions were kept in 20mM sodium phosphate, 150mM NaCl, pH=6 at 5°C for 24hrs. For wavelength spectra, measurements were made at every 0.5nm step with an average time of 4 sec at each wavelength. For temperature induced denaturation, the temperature was increased at an average rate of 0.1°C per min as previously described²⁹. Thermal transitions were monitored at 225 nm which is the characteristic maximum for the triple helix²⁹ using $c=0.35\text{mM}$ for (POG)₁₀, the hybrid peptide, and the GNNQQNY peptide. The melting temperature (T_m) is calculated as the temperature at which the fraction folded value is equal to 0.5²⁹.

Dynamic Light Scattering (DLS)

DLS measurements were performed using a Dyanopro Titan (Wyatt Technology Corp.) equipped with a Peltier temperature controller in a 12 μ l cuvette. Solutions were passed through 0.02 μ m syringe filters to remove any unwanted dust particles or pre-existing oligomers which might interfere with subsequent kinetic studies³⁰. All samples were equilibrated for 5 minutes at 20°C before measurements were performed. From then on, intensity autocorrelation functions of the scattered light were continuously measured using an acquisition time of 60 sec per correlation function. To obtain the hydrodynamic radius (R_h), the intensity correlation functions were analyzed using the Dynamic Software provided by Wyatt Technology. A viscosity value $\eta = 1.02\text{cp}$ was used for 20mM sodium phosphate, 150mM NaCl pH=6 at 20°C.

Electron Microscopy (EM)

Aggregated peptide samples were negatively stained and analyzed by electron microscopy using a JEOL 2100 electron microscope (Center for Nanoscale Systems, Harvard University), to visualize the morphology of higher order structures. Small aliquots (~5 μ l) of samples were absorbed onto carbon-coated grids for 30 sec and then one drop of 0.5% uranyl acetate was applied for 30 sec. The grids were washed twice with distilled water and dried at room temperature overnight.

Congo Red and Thioflavin T Binding by Fibrils

Congo red (C.I. No. 22120, Fluka Analytical, St. Louis, MO) was dissolved in deionized water and filtered through 0.2 μ m syringe filters. The absorption spectrum of 5 μ M of Congo red was measured alone, as well as with 10 μ l of either monomer or aggregates of peptides in 1 ml solution. The spectra of 10 μ l of monomers or aggregates were also measured without Congo red. Then, the spectrum of monomers/aggregates alone was subtracted from the spectrum of monomers/ aggregates with Congo red. For the ThT binding assay, ThT (Sigma-Aldrich) was dissolved in 20mM phosphate buffer, pH=6 with 150mM NaCl and filtered with 0.22 μ m syringe filters. The peptide solution was diluted to a final concentration of 10 μ M in buffer with 50 μ M ThT as described previously³¹. The fluorescence scan was performed on Fluoromax4 spectrofluorometer with excitation at 450nm and the emission scan from 465 to 600nm.

Nuclear Magnetic Resonance (NMR)

All samples were dissolved in 20 mM Sodium phosphate, 150 mM NaCl, at pH 6 with 10% D₂O, reaching concentrations of 0.15 to 0.5 mM for GNNQQNY and 0.7 mM for (GPO)₅GNNQQNY(GPO)₄. The data sets were processed using NMRPipe³² and spectra were assigned and analyzed using CARA³³ software. The residues within the GNNQQNY peptide are denoted as G¹N²N³Q⁴Q⁵N⁶Y⁷, while the notation (GPO)₅G¹N²N³Q⁴Q⁵N⁶Y⁷(GPO)₄ is used to distinguish the same residues within the hybrid peptide.

NMR experiments were acquired on a Bruker 600 MHz spectrometer and a Varian 600 MHz, equipped with a cryoprobe. The ¹H NMR spectrum of GNNQQNY (c=0.18mM) was monitored at 0, 4, and 24 hrs at 20°C and at 0, 2, 8, and 32 hrs at 0°C (c = 0.15 mM). The ¹H NMR spectrum of the hybrid peptide was acquired at 0°C for 6 days. Peptides assignments were achieved using a MLEV or DIPSI pulse sequence in a total correlation spectroscopy ([¹H-¹H]-TOCSY) experiment^{34,35} with a mixing time of 40 and 60 msec and a duration of 2 to 4 hrs, and Overhauser enhancement spectroscopy ([¹H-¹H] NOESY)³⁶ experiments with a mixing time of 50, 100, 150, 200, and 400 msec. In an attempt to distinguish between the monomeric and the trimer peaks in the hybrid peptide, one and two dimensional TOCSY experiments were conducted at 0 and 40°C.

Results

Peptides containing an amyloidogenic sequence and a collagen triple-helix

The peptides characterized in this study include a very stable triple helical peptide (POG)₁₀; a well characterized amyloid forming peptide GNNQQNY based on the yeast prion Sup35⁸; and a newly designed hybrid peptide which incorporates features from both systems. The hybrid peptide has the GNNQQNY sequence between two (GPO)_n sequences: (GPO)₅GNNQQNY(GPO)₄. Although many amyloid studies are carried out at low pH or in water, more physiological conditions were selected here, using phosphate buffered saline, to better mimic possible *in vivo* collagen-amyloid interactions. All conformation, stability, and aggregation studies were investigated under a set of standard conditions: 20mM phosphate,

150mM NaCl, pH=6 (PBS, pH 6). The value of pH=6, rather than pH 7, was selected because of the slower hydrogen exchange rates for NMR experiments.

(a) Circular Dichroism (CD) and Dynamic Light Scattering (DLS) studies—

Circular dichroism (CD) spectra and melting curves demonstrated that (POG)₁₀ formed a triple-helical structure under standard conditions used in this study (PBS, pH 6). The peptide GNNQQNY showed a CD spectrum with only a minimum near 195nm, as previously reported, consistent with a random coil structure⁸. The CD spectrum of the hybrid peptide showed triple helical features (maximum at 225nm, minimum at 198nm) but has a somewhat lower magnitude at 225nm ($MRE_{225nm} \sim 4300 \text{ deg cm}^2\text{dmol}^{-1}$), compared with (POG)₁₀ ($MRE_{225nm} \sim 5000 \text{ deg cm}^2\text{dmol}^{-1}$) (Fig. 1A). The ratio of positive (225nm) to negative (198 nm) peak magnitudes (rpn) in the CD spectra is a measure of triple helix formation³⁷, and the rpn = 0.12 value for the hybrid peptide is similar to that of (POG)₁₀ indicating a high triple helical content (Fig. 1A). In addition, triple-helix formation on both ends of the hybrid peptide was supported by the good agreement between the MRE_{225nm} expected for (POG)₉, and that obtained by subtracting the MRE_{225nm} of the GNNQQNY peptide from that of the hybrid peptide (data not shown).

CD studies of the thermal transition of the hybrid peptide indicated a $T_m = 23^\circ\text{C}$, which is substantially lower than seen for (POG)₁₀ ($T_m = 60^\circ\text{C}$) (Fig. 1B). The hybrid peptide had a sharp thermal transition, suggesting both the (GPO)_n terminal regions and the central GNNQQNY unfold cooperatively. The GNNQQNY peptide showed only a linear decrease in MRE_{225nm} with increasing temperature, consistent with the random coil structure prediction.

All peptides were soluble for at least several hours under these conditions (20°C , $c = 0.5\text{mM}$), and dynamic light scattering (DLS) measurements showed a single particle population, consistent with no higher order structure initially at this low concentration. The hydrodynamic radius of each peptide is consistent with a single molecule: $R_h = 1.9 \text{ nm}$ for (POG)₁₀; $R_h = 1.1 \text{ nm}$ for GNNQQNY; $R_h = 2.9 \text{ nm}$ for (GPO)₅GNNQQNY(GPO)₄ (“Fig.S1 in the Supporting Material”).

(b) NMR studies on peptides—NMR studies were used to further investigate the conformational features of the GNNQQNY peptide. A combination of TOCSY and NOESY spectra allowed the complete proton assignment of the 7 spin systems observed for GNNQQNY. The chemical shifts of the alpha protons were typical of disordered peptides/proteins at 20°C with a chemical shift deviation less than 0.15 ppm³⁸⁻⁴⁰, consistent with the disordered CD spectrum and the absence of any thermal transition.

The 2D TOCSY spectrum of the GNNQQNY peptide showed additional spin systems in the amide region with lower peak intensity, and these had the same chemical shifts values as Q⁵, N⁶ and N⁷, except for the chemical shift of their amide protons (Fig. 2A). The sensitivity of amide protons to peptide/protein conformational changes supported assignment of these 3 additional spin systems to a minor population of Q^{5*}, N^{6*} and Y^{7*} residues. These additional peaks were less pronounced in the spectrum acquired at 0°C , where aggregation is slower, compared with 20°C .

The hybrid peptide (GPO)₅GNNQQNY(GPO)₄ was also characterized by NMR. The absence of sequential cross peaks in the NOESY spectra did not allow residue assignments. However, the TOCSY spectrum presented spins systems which could be assigned to residues within the central sequence, in addition to spin systems from the flanking GPO sequences (Fig. 2B). The TOCSY spectrum of the hybrid peptide showed one spin system for each of the six central residues N²/N³ Q⁴ Q⁵ N⁶ Y⁷ (Fig. 2B), which could be assigned

based on the GNNQQNY peptide spectrum (Note that the prime numbers refer to residues within the hybrid; Table 1). The amide protons of the internal residues $N^3Q^4Q^5N^6$ showed the same chemical shifts as seen in the unstructured GNNQQNY peptide, consistent with their being in a disordered state, while chemical shift differences were seen for the amide protons from the outermost residues $G^1N^2NQQNY^7$ (upfield shift of -0.14 ppm of N^2 and a downfield shift of 0.09 ppm of Y^7). Given the numerous Gly residues in the GPO termini, it was not possible to assign a specific resonance to G^1 , but most of the Gly resonances seen in the hybrid peptide were downfield shifted relative to G^1 in the GNNQQNY peptide. The upfield chemical shift of the alpha-protons of N^2 (-0.05 ppm) is consistent with a conformational change from a disordered to a helical structure at this residue. The alpha-protons of residues $N^3Q^4Q^5N^6Y^7$ showed a downfield chemical shift, which could be indicative of beta-sheet propensity. The Tyr residues showed two different chemical shifts in the aromatic region, which might be indicative of two orientations of the phenolic ring. This was not present in the GNNQQNY peptide. However, different phenolic ring orientations of Tyr, caused by 2-fold 180° flips at an intermediate exchange rate, have also been observed by solid state NMR in GNNQQNY peptide and were linked to the amyloid fibrils and orthorhombic crystals formation (being absent in monoclinic crystals²⁵). These might indicate structural differences in the packing of the GNNQQNY and hybrid peptides within the amyloidogenic structures. Aromatic residues have been suggested by others to play special role in the formation and/or stabilization of amyloid-like fibrils⁴¹⁻⁴³.

In addition to the central GNNQQNY sequence, the hybrid peptide has 9 Pro, 9 Hyp, and 10 Gly residues in each chain, but due to extensive overlap of the resonances in the repeating tripeptides units, only 4 spins systems for Pro or Hyp, and 6 spin systems for Gly residues could be identified. Out of the 6 spin systems assigned to Gly, there is evidence that at least 3 of them belong to Gly in the triple helical conformation. When the temperature was increased from 0°C to a temperature higher than the melting temperature (40°C), two Gly resonance peaks in the amide region disappeared, consistent with an assignment to the trimer conformation. In addition, one of the other resonances (H_N 8.60 ppm, $H_{\alpha 1}$ 4.22 ppm, and $H_{\alpha 2}$ 4.22 ppm) matched the chemical shift observed for a Gly residue trimer resonance in $(\text{POG})_{10}$ assigned to the terminus because of its decreased order compared with internal trimer Gly residues⁴⁴. It was not possible to conclude whether Gly^1 is ordered in the hybrid peptide trimer.

Aggregation of the GNNQQNY peptide

Light scattering intensity was used to study the aggregation kinetics of peptide GNNQQNY at different concentrations. These aggregation experiments were carried out at 20°C (PBS, pH=6); a higher temperature might jeopardize the triple-helix nature of some of the peptides ($T_m = 26.9^\circ\text{C}$ for the positively charged GROGKRGKO peptide and $T_m = 23^\circ\text{C}$ for the hybrid peptide) and the aggregation at lower temperatures (e.g. 10°C) was too slow. At 20°C no aggregation was observed within 24 hrs for concentrations less than 0.3mM (Fig.3A), while at higher concentrations, an increase in scattering intensity was observed with time, indicating self-association of the molecules. The kinetics followed the expected nucleation-growth mechanism, showing a defined lag phase and then a growth phase, followed by a plateau, where fibrils come out of solution. For the aggregation process, increasing concentration resulted in a decreased lag time and increased rate of growth (Fig.3A). For example, the lag time decreased from 6 to 2hrs when the concentration increased from 0.4 to 0.5mM . At concentrations above 0.6mM , the growth phase was seen at the earliest time points, with no observable lag phase. After aggregation was complete, the particles settled down out of the light path, resulting in decreased scattering intensity.

Increasing temperature accelerated the aggregation of peptide GNNQQNY. At low temperature $T=5^{\circ}\text{C}$, aggregation was observed at concentrations above 0.55mM only after long time periods (e.g 16 hrs for $c=0.6\text{mM}$) (data not shown). Higher temperatures led to a decreased lag time and increased rate of growth. At 20°C , light scattering of a 0.5mM peptide solution showed only a single particle with $R_h=1.1\text{nm}$ initially, but after 2hrs, hydrodynamic radii representing larger particles around $R_h=100\text{nm}$ were also observed. At later stages, larger particles in the μm range were observed (“Fig.S1 in the Supporting Material”).

To determine whether the aggregates formed were amyloid-like, Congo red and Thioflavin T binding assays were used. Addition of GNNQQNY aggregates to Congo Red demonstrated a shift of the 514nm absorbance characteristic of amyloid fibrils (Fig. 4A). This spectral shift was not observed when the soluble, non-aggregated form of the peptide was added to Congo red. A thioflavin T (ThT) binding assay showed an increase in fluorescence intensity at 482nm upon addition of GNNQQNY in its aggregated, but not its soluble form, supporting presence of amyloid-like structures (Fig.4B). The increase in fluorescent intensity was smaller for aggregates formed in our standard PBS, pH 6 buffer than seen for aggregates formed in water (Fig. 4B).

Electron microscopy was used to visualize the structure of aggregates formed by peptide GNNQQNY (Fig.5A), after negative staining. Although straight unbranched amyloid fibrils were reported when GNNQQNY aggregates from water^{8,22}, aggregates formed in our PBS, pH 6 buffer showed worm-like or network-type fibrous structures, similar to the morphology reported for some protofibrils or amyloid structures^{6,45,46}.

Self-association of (POG)₁₀ and the hybrid peptide

The self-association of (POG)₁₀ was studied previously using turbidity measurements in PBS at pH=7, and it was shown that the critical concentration is $\sim 1\text{mM}$ and the critical temperature is $\sim 35^{\circ}\text{C}$ ¹⁸. In this study, light scattering was used to study the aggregation kinetics of (POG)₁₀ in 20mM Sodium phosphate, 150mM NaCl at pH = 6, 20°C , 0.5mM (Fig. 3B). The hydrodynamic radius of the (POG)₁₀ molecule is $R_h=1.9\text{nm}$, and no aggregation was detectable for the first 20hrs. After $\sim 24\text{hrs}$, the DLS showed a broader distribution with $R_h=4\text{nm}$, suggesting an increased particle size distribution, e.g. molecules self-associating into oligomers (Fig.S1 in the supporting material). At higher concentrations ($\sim 1\text{mM}$) and higher temperatures (35°C), visible aggregates were apparent within a few hours. Some aggregation was also apparent at low temperature (4°C) at high concentration ($\sim 2\text{mM}$) after long time periods. The (POG)₁₀ aggregates formed did not show any spectral shift in a Congo red binding assay confirming that (POG)₁₀ aggregates did not form any cross β structure (Fig.4D). Electron microscopy of (POG)₁₀ aggregates showed some irregular structures, with no sign of any fibrillar or ordered structures (Fig.5C).

To study the aggregation kinetics of the hybrid peptide, (GPO)₅GNNQQNY(GPO)₄ light scattering was measured at different concentrations (20°C). No aggregation was observed within 24h for concentrations below 0.4mM (data not shown), but above this concentration, an increase in scattering intensity was observed with time, indicating self-association of the molecules (Fig.3B). The lag phase of the hybrid peptide was substantially longer than observed for the GNNQQNY peptide (15 hrs vs. $\sim 2\text{hrs}$). Light scattering of a 0.5mM peptide solution maintained at 20°C for 2 hrs showed only a single particle size with $R_h=2.9\text{nm}$, but after 17hrs, hydrodynamic radii representing larger particles of several hundred nanometers were also observed (“Fig.S1 in the Supporting Material”). Decreasing temperature slowed aggregation; after 6 days at low temperature $T=5^{\circ}\text{C}$, no aggregation was observed at $c=0.5\text{mM}$, with DLS showing only a single particle size, $R_h\sim 3\text{nm}$.

Addition of the hybrid peptide aggregates to Congo red demonstrated the shift of the 514nm absorbance characteristic of amyloid fibrils (Fig.4C), indicating the GNNQQNY sequence is still capable of promoting amyloid-like aggregation even when surrounded by triple-helices. This spectral shift was not observed when the soluble, non-aggregated form of the hybrid peptide was added to Congo red (Fig.4C).

Electron microscopy was used to visualize the structure of aggregates formed by the hybrid peptide (Fig.5B). Network-like fibrous structures were observed and their morphology looks very similar to that of the GNNQQNY peptide (Fig. 5B), except that fewer structures were detected.

Effect of collagen-like peptides on GNNQQNY aggregation

As described above, the GNNQQNY peptide formed amyloid-like aggregates at 20°C (PBS, pH 6), following a nucleation-growth mechanism, and collagen model peptides were added to the GNNQQNY peptide under these conditions to see if they affected amyloidogenesis.

(a) Addition of (POG)₁₀—Light scattering measurements were performed after incremental addition of (POG)₁₀ molecules to a fixed concentration of GNNQQNY peptide (0.5mM at 20°C) (Fig.3C). When (POG)₁₀ was added in a very small quantity to GNNQQNY (1:200 molar ratio), no effect was seen on the aggregation of the GNNQQNY peptide. However, when the molar ratio reached 1:20 (POG:GNNQQNY) and higher, addition of (POG)₁₀ significantly inhibited the self association of the GNNQQNY peptide, increasing the lag time and decreasing the rate of growth of fibrils. At 1:2 POG:GNNQQNY ratio, c=0.5mM, the lag time increased from 2 to ~8 hrs, and the slope of the growth curve decreased as well.

Electron microscopy was used to visualize the structure of aggregates formed after addition of (POG)₁₀ to GNNQQNY in a 1:2 ratio. The network fibrous structures observed had morphology very similar to that seen for GNNQQNY and the hybrid peptide, but the fibrils were very sparse (data not shown).

(b) Addition of a non-triple-helical peptide, (POG)₁₀ with a single Gly deletion—Since addition of (POG)₁₀ to the GNNQQNY peptide (0.45mM) in a 2:1 ratio increased the lag time from 4 hrs to 11 hrs, and delayed amyloid fibril growth, experiments were pursued to see if the triple-helix conformation is a critical factor in this effect. A peptide with a single Gly deletion in the (POG)₁₀ sequence was previously shown to be unable to form a triple-helical structure²⁸, and this peptide (POG)₄PO(POG)₅ was added to the GNNQQNY peptide (c=0.45mM) at 20°C in a 1:2 ratio. In contrast to (POG)₁₀, the monomer non-helical peptide did not delay the lag phase but surprisingly, it did slow down the growth rate in a manner similar to (POG)₁₀ (Fig. 3D).

(c) Addition of a positively charged triple-helical peptide—Since a previous report suggested that a highly positively charged region of the Type XXV collagen triple-helix was essential for binding to amyloid and inhibiting elongation⁴⁵, a peptide with many positive charges was also studied. The peptide chosen was (GPO)₂GROGKRGKO(GPO)₃GG, a peptide with a heparin binding motif that was previously reported to form a triple-helical structure with a T_m of ~26.9°C²⁷. Addition of this basic peptide to GNNQQNY (c=0.45mM) at 20°C in a 1:2 ratio increased the lag phase (from ~4 hrs to ~7 hrs), but this delay was less than seen for (POG)₁₀. In addition, the growth rate was slower, similar to that seen for both other collagen-like peptides (Fig.3D). Neither the Gly deletion peptide nor the positively charged collagen model peptide showed any sign of aggregation after 48hrs (T=20°C; c=0.25mM) (data not shown).

Discussion

The study of model peptides may help clarify the relation between collagen and amyloid fibrils, since there is evidence from a variety of approaches that type XXV collagen can bind to amyloid *in vitro* and may play a role in the development of amyloid plaques and Alzheimers Disease^{1-6,12,45}. The experiments described here investigate the effect of collagen model peptides on the aggregation of an amyloidogenic peptide and also characterize aggregation when a well defined and highly amyloidogenic sequence GNNQQNY is inserted between strong triple-helix promoting (Gly-Pro-Hyp)_n sequences to form a hybrid peptide.

Circular dichroism spectroscopy and NMR spectroscopy showed the GNNQQNY peptide forms a random coil structure in solution (PBS, pH 6), while the hybrid peptide contained a central disordered region transitioning to triple-helices on both ends. The propensity of GNNQQNY to form higher order aggregates is delayed when the sequence is placed within a triple-helix context, which appears to be a consequence of conformational constraints on its outer residues near the Gly-Xaa-Yaa sequences. The G^{1'}, N^{2'}, and Y^{7'} residues at the amyloid/triple-helix junction showed chemical shifts that differ from those of the corresponding residues within the small GNNQQNY peptide. Attachment of these terminal residues of the inserted GNNQQNY to a rigid triple-helix structure is likely to induce conformation constraints that prevent their adopting a fully disordered random state. Such constraints may disfavor a conformational change that must occur to promote amyloidogenesis. The hybrid peptide shows a significant propensity to self-associate close to its melting point, but not at lower temperature. Near the melting temperature, the triple helix in the hybrid peptide might loosen and provide flexibility to the central amyloidogenic sequence, allowing it to transition to a conformation that promotes aggregation. Thus, the aggregation propensity of any amyloidogenic sequences within interruption sites in non-fibrillar collagens, such as GEFYFDLRLK within type IV collagen¹³, is likely to be moderated by the surrounding triple-helix.

To model the effect of type XXV and other collagens on amyloid fibril formation, the peptide (POG)₁₀ was added to the GNNQQNY peptide under associating conditions. Addition of (POG)₁₀ peptide increased the lag phase and slowed down the formation of amyloid aggregation. The increased lag phase indicates that (POG)₁₀ delays the nucleation step. Comparison with other collagen model peptides demonstrated the triple-helix conformation is required for this nucleation delay and less delay is seen for a positively charged triple-helical peptide than for (POG)₁₀. All of the Gly-Xaa-Yaa peptides, including the monomer form, slowed amyloid aggregation. It is interesting to speculate on the mechanism by which collagen-like peptides can affect amyloid nucleation and growth. The high imino acid content of the collagen peptides is very unfavorable for beta strand structure⁴⁷, so it is unlikely that these peptides could participate in a beta sheet to inhibit a nucleating or growing GNNQQNY strand⁴⁸. Within each Gly-Pro-Hyp tripeptide unit of the triple-helix, there is one direct hydrogen bond (Gly NH...ProC=O), leaving two unsatisfied backbone C=O groups, which can interact with water⁴⁹. We hypothesize that these two available C=O groups in Gly-Pro-Hyp units of a triple-helix can bind to backbone amides or to Gln or Asn side chains of GNNQQNY, slowing its nucleation step. It was proposed⁴⁵ that a very basic triple-helix region is responsible for inhibition of amyloid growth, but our observations indicate that (POG)₁₀ leads to a greater nucleation delay and a similar inhibition of growth as a positively charged triple-helical peptide. In the positively charged central region of GROGKRGKO peptides, one of the backbone C=O groups forms a water mediated hydrogen bond to a backbone amide⁵⁰⁻⁵², leaving only one carbonyl available, which may result in a weaker interaction and less nucleation delay of GNNQQNY. The inhibition of the amyloid aggregation stage appears less specific, since it

doesn't require a triple-helix. We suggest that imino acid-aromatic CH... π interactions between the Pro/Hyp within a collagen peptide and the Tyr within the amyloidogenic peptide may play a role. Previous studies showed the importance of Tyr residues in stabilizing the initial parallel aggregates of GNNQQNY⁴², and Tyr-imino acid interactions have been implicated in interactions between triple-helical molecules¹⁷. Type XXV collagen was reported to alter the growth/elongation phase of amyloid, but not affect nucleation. The peptides studied here all contain repeating Gly-Pro-Hyp sequences which are not found in native type XXV collagen, consistent with the hypothesis that it is the steric features of the two available C=O moieties in repeating Gly-Pro-Hyp sequences within the triple-helix that interact with GNNQQNY and delay its nucleation.

The results on peptides confirmed the interaction between these two distinct protein structures, which are both found in the extracellular matrix. Thus, the triple-helix has an inhibitory effect on the rate of amyloidogenesis when it is added to the system independently, as well as when it is flanking the amyloidogenic sequence within the same polypeptide chain. These peptide results are consistent with an anti-amyloidogenic role for the type XXV and possibly other collagens, but further investigation on the nature of the molecular interactions and mechanism of inhibition may be useful in designing anti-amyloidogenic molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(Pro-Hyp-Gly) ₁₀	(POG) ₁₀ ; Standard amino acids codes are used, with the addition of hydroxyproline designated by Hyp (3-letter code) and O (1-letter code)
R _h	hydrodynamic radius
T _m	melting temperature

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Figure 1.
(A): Circular Dichroism (CD) spectra and (B) CD melting curve of (POG)₁₀ (—), GNNQQNY (---), and the hybrid peptide (····) at 0°C in 20mM Sodium phosphate buffer, pH=6 with 150mM NaCl.

Figure 2.

Amide region of the 2D TOCSY spectrum of (A) $G^1N^2N^3Q^4Q^5N^6Y^7$ peptide (0.18 mM) and (B) $(GPO)_5G^1'N^{2'}N^{3'}Q^{4'}Q^{5'}N^{6'}Y^{7'}(GPO)_4$ peptide (0.7 mM), at 0°C in PBS at pH 6. Note that the primed numbers refer to the GNNQQNY residues in the hybrid peptide while the non-primed numbers refer to these residues in the GNNQQNY peptide. The seven spin systems corresponding to the major population of GNNQQNY peptide are labeled in black, while the three minor spin systems (Q^{5*} , N^{6*} , and Y^{7*}) are labeled in red. In the hybrid peptide, the glycine peaks corresponding to the monomer and trimer state are denoted with a subscript M or T. For the remaining residues ($N^{2'}N^{3'}Q^{4'}Q^{5'}N^{6'}Y^{7'}$) only the monomer peak was observed. The green residues and boxes highlight the shift of N^2 and Y^7 residues in the GNNQQNY peptide relative to the hybrid peptide, $N^{2'}$ and $Y^{7'}$, respectively; these are not boxed in the hybrid spectrum due to peak overlap.

Figure 3.

The overall scattering intensity vs. incubation time of (A) GNNQQNY at different concentrations in 20mM Sodium Phosphate, 150mM NaCl, pH=6 at 20°C; (B) Comparison of (POG)₁₀, GNNQQNY, and the hybrid peptide at c=0.5mM, 20°C (C) after incremental addition of (POG)₁₀ to fixed concentration (c=0.5mM) of GNNQQNY peptide at 20°C, at varying molar ratios of GNNQQNY: (POG)₁₀ (20:1 and 2:1); (D) GNNQQNY (0.45mM) alone (●) and with the addition of 0.225mM (POG)₄PO(POG)₅ (□); 0.225mM (GPO)₂GROGKRGKO(GPO)₃GG (■) or 0.225mM (POG)₁₀ (○) in 20mM Sodium phosphate buffer, pH=6 with 150mM NaCl at T=20°C.

Figure 4.

(A) Absorption spectra of Congo red alone (—) and after addition to soluble GNNQQNY peptide (·····), and aggregates (---) of GNNQQNY; (B) Fluorescence emission spectra of ThT alone (—), and with soluble GNNQQNY in PBS (·····); aggregated GNNQQNY in PBS (---); soluble GNNQQNY peptide in water (·····); and aggregated GNNQQNY peptide in water (---); (C) Congo red with hybrid peptide, Congo red alone (—) and after addition to soluble hybrid peptide (·····) and aggregates (---); and (D) Congo red with (POG)₁₀, Congo red alone (—) and after addition to soluble (POG)₁₀ peptide (·····) and aggregates (---).

Figure 5. Electron Micrograph specimens of negatively stained specimens of (A) GNNQQNY peptide, (B) Hybrid peptide, and (C) (POG)₁₀.

¹H NMR assignments for Ac-G¹N²N³Q⁴Q⁵N⁶Y⁷-NH₂ and Ac-(GPO)₅G¹N²N³Q⁴Q⁵N⁶Y⁷-(GPO)₄-NH₂ at 0 or 20°C, and respective chemical shift differences at 0°C (ppm related to DSS), with the most significant changes of the amide and alpha protons indicated by an asterisk. The chemical shifts of the minor population observed in the amyloidogenic spectra are represented in parentheses. The slash mark is used to indicate the two distinct resonances of the Y⁷ phenolic protons.

Table 1

G ¹ N ² N ³ Q ⁴ Q ⁵ N ⁶ Y ⁷				(GPO) ₅ G ¹ N ² N ³ Q ⁴ Q ⁵ N ⁶ Y ⁷ -(GPO) ₄			
		20°C		0°C		0°C	
Residue	atom	δ (ppm)		Residue	atom	δ (ppm)	Δσ (ppm)
G ¹	HN	8.32	8.43				
	HA	3.92	3.90				
N ²	HN	8.47	8.57	N ²	HN	8.43	-0.14*
	HA	4.69	4.71		HA	4.66	-0.05*
HB ²		2.83	2.79		HB ²	2.80	0.01
	HB ³	2.78	2.72		HB ³	2.74	0.02
HD ²¹		7.54	7.62		HD ²¹	7.76	0.14
	HD ²²	6.88	6.96		HD ²²	7.30	0.34
N ³	HN	8.52	8.62	N ³	HN	8.64	0.02
	HA	4.70	4.67		HA	4.68	0.01
HB ²		2.85	2.84		HB ²	2.83	0.00
	HB ³	2.77	2.74		HB ³	2.74	0.00
HD ²¹		7.61	7.69		HD ²¹	7.69	-0.01
	HD ²²	6.93	7.01		HD ²²	7.04	0.03
Q ⁴	HN	8.43	8.43	Q ⁴	HN	8.44	0.01
	HA	4.22	4.22		HA	4.23	0.02
HB ²		2.09	2.09		HB ²	2.10	0.02
	HB ³	1.95	1.95		HB ³	1.96	0.02
HG		2.30	2.30		HG	2.32	0.01
	HN	8.47 (8.53)	8.47 (8.65)	Q ⁵	HN	8.47	0.00
HA		4.18 (4.27)	4.18 (4.21)		HA	4.20	0.02
	HB	1.89 (1.90)	1.89		HB	1.90	0.01
HG		2.23 (2.25)	2.23		HG	2.28	0.05

$G^1N^2N^3Q^4Q^5N^6Y^7$				$(GPO)_5G^1N^2N^3Q^4Q^5N^6Y^7(GPO)^4$			
		20°C		0°C		0°C	
Residue	atom	δ (ppm)				δ (ppm)	$\Delta\sigma$ (ppm)
N ⁶	HN	8.36 (8.41)	8.45 (8.52)		N ^{6'}	HN	8.44
	HA	4.65 (4.66)	4.63 (4.64)			HA	4.66
	HB2	2.74 (2.74)	2.72 (2.72)			HB2	2.73
	HB3	2.65 (2.67)	2.63 (2.63)			HB3	2.61
	HD21	7.47	7.54			HD21	7.54
Y ⁷	HD22	6.86	6.94			HD22	6.96
	HN	8.17 (8.22)	8.30 (8.37)		Y ^{7'}	HN	8.38
	HA	4.53 (4.53)	4.49 (4.49)			HA	4.58
	HB2	2.91 (2.91)	2.89 (2.86)			HB2	2.85
	HB3	3.11 (3.12)	3.09 (3.10)			HB3	3.09
	HD	7.14	7.11			HD	7.10/7.28
	HE	6.83	6.80			HE	6.80/6.88
							0.00