Conformational Transitions of the Crosslinking Domains of Elastin During Self-assembly*

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Background: Elastin is a polymeric protein providing extensibility and elastic recoil to tissues.

Results: Crosslinking domain structure shifts from random coil to β-strand to α-helix during assembly of elastin matrix.

Conclusion: Crosslinking domains have a previously unappreciated structural lability during assembly, which is highly susceptible to mutations of lysine residues.

Significance: Understanding of differentiation between elastin self-assembly and amyloid-like aggregation.

ABSTRACT

Elastin is the intrinsically disordered, polymeric protein imparting the exceptional properties of extension and elastic recoil to the extracellular matrix of most vertebrates. The monomeric precursor of elastin, tropoelastin, as well as polypeptides containing smaller subsets of the tropoelastin sequence, can self-assemble through a colloidal phase separation process called coacervation. Present understanding suggests that self-assembly is promoted by association of hydrophobic domains contained within the tropoelastin sequence, while polymerization is achieved by covalent joining of lysine side chains within distinct alanine-rich, α-helical crosslinking domains. In this study, model elastin polypeptides were used to determine the structure of crosslinking domains during the assembly process and the effect of sequence alterations in these domains on assembly and structure. CD temperature melts indicated that partial α-helical structure in crosslinking domains at lower temperatures was absent at physiological temperature. Solid-state NMR demonstrated that β-strand structure of the crosslinking domains dominated in the coacervate state, although α-helix was predominant after subsequent crosslinking of lysine side-chains with genipin. Mutation of lysine residues to hydrophobic amino acids, tyrosine or alanine, leads to increased propensity for β-structure and the formation of amyloid-like fibrils, characterized by ThT binding and transmission electron microscopy. These findings indicate that crosslinking domains are structurally labile during assembly, adapting to changes in their environment and aggregated state. Furthermore, the sequence of crosslinking domains has a dramatic effect on self-assembly properties of elastin-like polypeptides, and the presence of lysine residues in these domains may serve to prevent inappropriate ordered aggregation.
(1,2). Tropoelastin is a highly repetitive 60 kDa protein consisting of an alternating arrangement of hydrophobic and crosslinking domains. Hydrophobic domains are rich in non-polar amino acids such as proline, glycine, valine and leucine. These domains have been shown by experiment and molecular dynamics simulations to be highly disordered and flexible in solution (3-9). The high entropy and hydrophobic character of these domains is believed to be responsible for the extensibility and restoring force of polymeric elastin (6,10-12).

In contrast, crosslinking domains stabilize the polymeric structure of elastin through covalent crosslinking of lysine side chains. Crosslinking is achieved in vivo through oxidative deamination of the lysine side chains by the enzyme lysyl oxidase, with subsequent condensation linking 2, 3 or 4 side chains (13,14). Crosslinking domains can be separated into two specific sequence types, those consisting of lysines located within a proline-rich sequence (KP-type), or the more abundant type that contain polyalanine sequences interspersed with lysines in the form of KAAK or KAAAAK (KA-type). KA-type crosslinking domains have been suggested to form an α-helical secondary structure that is believed to facilitate formation of crosslinks by bringing lysine residues together on the same face of the helix (4,15-18).

Despite the complex relationship in vivo between tropoelastin and other matrix-associated proteins, we and others have shown that both tropoelastin and smaller polypeptides based on the sequence of tropoelastin can self-assemble in vitro independently of other proteins through a characteristic heat or salt-induced liquid-liquid phase separation process called coacervation (17,19-23). Coacervation is an endothermic, entropically driven process (24-26) through which hydrophobic domain interactions have been suggested to concentrate and align crosslinking domains for polymerization (5,22,27,28). In contrast to other forms of protein aggregation that create highly ordered insoluble structures, such as β-strand stacking in amyloid fiber formation, coacervation forms viscous protein-rich colloidal droplets that can interact and coalesce (29,30). Unlike amyloid fibril formation, coacervation may be reversed in vitro by immediately lowering the temperature of the colloidal suspension (20,30). To this point, very little is known about the detailed structure of tropoelastin or elastin-like polypeptides in the coacervate form.

Coacervates from both full-length tropoelastin and smaller elastin-like polypeptides can be crosslinked using crosslinkers that react with amino groups of lysine side chains. The resulting insoluble materials have elastic properties comparable to those of native polymeric elastin (22,28,31). This finding, along with the ability of coacervate droplets to self-organize into extended network structures, suggests that the basic information needed for elastin assembly and elastomeric properties is provided by sequence motifs in the primary amino acid sequence of tropoelastin. As a result, we and others have attempted to determine the special properties specific to the sequence of tropoelastin that promote self-assembly into an elastic network, and that differentiate it from other types of ordered aggregation such as amyloid formation.

Although isolated hydrophobic domains and synthetic polypeptides consisting only of tandem repeats mimicking hydrophobic domain sequences will undergo coacervation, crosslinking domains do not coacervate on their own (19). As a result, the current model for elastin assembly considers the crosslinking domains as relatively passive participants in the process, maintaining α-helical structure throughout assembly from monomer to coacervate to crosslinked polymer. However, formation of lysine-derived crosslinks by these KA-type crosslinking domains is strongly conserved in elastins across vertebrate phylogeny (32-34), suggesting these sequences provide particular advantages to assembly. Furthermore, although they do not coacervate on their own, we and others have shown that the sequence and arrangements of the crosslinking domains within-elastin-like polypeptides can have a dramatic effect on their coacervation characteristics (17).

In spite of the general acceptance of the α-helical character of KA-type crosslinking domains, the stability of these helices has been questioned. Observed helical content, measured by CD or NMR, is consistently lower than would be expected for fully-formed helical structures (4,16,17,35). Indeed, fully formed α-helices were only observed on the addition of known helix-inducing solvents such as trifluoroethanol (TFE) (4,16). Thus, it remains unclear whether interaction of lysine side chains in the crosslinking
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domains is entirely dependent on alignment of hydrophobic domains or may be the result of stochastic fluctuations in structure that lead to juxtaposition of lysine side chains.

In this study we use simple elastin-like polypeptides to investigate the effect of sequence and environment on the detailed structure of KAK-type crosslinking domains. We demonstrate that the structures of these domains have a significant but previously unrecognized dependence on sequence, and that they undergo major structural transitions during the process of self-assembly.

EXPERIMENTAL PROCEDURES

Cloning and site directed mutagenesis— A PCR amplified DNA fragment consisting of exons 20, 21, 23 and 24 of human tropoelastin was cloned into the BamHI-EcoRI restriction sites of the T7 expression vector pET-32b (Invitrogen). This corresponds to the native sequence of human tropoelastin since exon 22, while present in the genomic sequence, is not expressed. Site directed mutations were created by engineering reverse or forward primers containing the mutation of interest and amplifying a short fragment of exons 20-24. These fragments were purified on agarose gels and used as primers for a subsequent PCR reaction with an additional forward or reverse primer to amplify the entire exon 20-24 sequence containing the mutation or mutations of interest. The product was cloned into the pET-32b vector as described above.

Polypeptide expression— The pET-32b expression constructs were used to transform BL21(DE3) competent cells. For unlabeled expression, transformants were grown at 37°C with shaking in 1 L of terrific broth liquid media supplemented with 100 µg/ml ampicillin and 2% glucose as the carbon source. IPTG concentrations were reduced to 50 µM and expression times were extended to 5 hours in minimal medium.

Polypeptide purification— Cell pellets were dissolved in 20 ml of 70% formic acid containing 1 g of CNBr and incubated at room temperature for 16 to 24 hours. The CNBr-treated cell solution was placed in dialysis tubing with a 3.5 kDa exclusion limit and dialyzed against 4 L of 20 mM sodium acetate pH 5.3, exchanging the buffer 4 times over a 48 hour period. To collect the supernatant, the cell debris was pelleted by centrifugation for 15 minutes at 12,000 x g. The polypeptide of interest was further purified by ion exchange FPLC and a final step of C4 reverse phase HPLC as described previously (17). MS was used to verify the expected molecular weights of the polypeptide products (Advanced Protein Technology Centre, The Hospital for Sick Children). The purified polypeptide was lyophilized and stored dry at room temperature. When dissolved in aqueous buffers the polypeptide concentrations were determined by UV absorption at 215 and 225 nm (36).

CD spectroscopy— CD experiments were carried out using a Jasco J-810 spectrometer. CD scans were performed in a 1.0 mm cuvette dissolving elastin polypeptides to 10 µM in 50 mM sodium phosphate, pH 7.0. The reported spectra are the mean of three stepwise scans between 260 and 190 nm, signal averaged for at least 2 s at each wavelength.

Thioflavin-T binding assays— Thioflavin-T (ThT) fluorescence was monitored using an Aviv 105 spectrofluorometer with excitation and emission wavelengths of 450 and 482 nm, respectively. Fresh ThT was dissolved to a final concentration of 10 µM in all samples. As a positive control, Aβ1-28 amyloid peptide was dissolved in 10 mM sodium phosphate, pH 6.0. All elastin polypeptides were dissolved in 10 mM sodium phosphate, pH 8.0.

Thioflavin-T microplate assays— Binding assays were carried out using a Molecular Devices SpectraMax Gemini EM microplate spectrofluorometer with excitation and emission wavelengths of 450 and 482 nm, respectively. Aliquots of 20 µl were taken daily from 200 µl of 50 µM polypeptide samples incubating at 37°C
and diluted to 5 µM in PBS, pH 7.4, containing a final concentration of 10 µM ThT.

Coacervation of polypeptides— Coacervation was measured as a light scattering at 440 nm using a UV-2401PC spectrophotometer (Shimadzu, Mandel Scientific) as described previously (30). Briefly, polypeptides were dissolved to 50 µM in PBS, pH 7.4, with additional NaCl to a final concentration of 1.5 M at a temperature approximately 5-10 °C below the coacervation temperature. Solution temperature was then raised at a rate of 1 °C/minute. Coacervation temperature was measured as the temperature of the initial upward inflection of the absorbance curve.

Light microscopy— Self-assembly of elastin polypeptides at a concentration of 50 µM was monitored in real time using a Zeiss Axiovert 200 inverted epifluorescence microscope with a temperature controlled Attofluor cell chamber (Molecular Probes). NaCl was added to a final concentration of 1.5 M for polypeptides EP20-24 and EP:K to A. NaCl was added to a final concentration of 1 M for polypeptide EP:K to Y. Sample chamber temperature was held constant at 5 °C above the coacervation temperature. All samples were imaged for 90 minutes, with snapshots taken after 60 minutes of incubation using differential interference contrast mode.

Transmission Electron Microscopy— To prepare specimens for electron microscopy, 4 µl of protein suspension (coacervate or fibrils) was diluted to 0.01 to 0.05 mg/ml and absorbed onto freshly glow-discharged carbon films prepared from copper rhodium grids (Electron Microscopy Sciences). The grids were washed in 10 µl filtered distilled water, blotted with filter paper, stained using 10 µl of freshly filtered 2% uranyl acetate and blotted a final time. The grids were then air dried at 20 °C. TEM images were obtained using a JEOL 1011 microscope operating at 80keV.

Solid-State NMR measurements— Coacervate samples of elastin-like polypeptides isotopically labeled with 13C and 15N for NMR measurement were prepared by incubating 1 mM polypeptide solution in PBS pH 7.4 with 1.5 M NaCl at 37 °C for 3 days. Polymerized materials labeled with 13C and 15N were prepared by genipin crosslinking as described previously (31). Lyophilized protein was packed into 22 µl 3.2 mm magic angle spinning (MAS) rotors. Hydrated coacervate or fibril samples were centrifuged and the pellets washed with water to remove salt then packed into 36 µl 3.2mm rotors with watertight seals.

Solid-state NMR measurements were performed using a triple-resonance 3.2 mm T3 MAS probe in a Varian VNMRS spectrometer operating at an 1H frequency of 499.70 MHz. Temperature control was obtained using high flow rates of cooled dry air on the sample, with temperature at the sample calibrated to 35 °C using the 207Pb chemical shift of Pb(NO3)2 (37). All spectra were externally referenced to the downfield 13C resonance of adamantane at 38.56 ppm relative to tetramethylsilane (38).

Experiments were performed at an MAS frequency of 11 kHz. Cross polarization (CP) was implemented using radio frequency (rf) field strengths of 40-60 kHz (13C) and 50-80 kHz (1H), a linear ramp on the 13C channel and contact times of 1 to 1.5 ms. π/2 pulse widths on all channels were 2.0-4.0 µs and 1H decoupling fields of 100 kHz were applied during all t1 and t2 acquisition periods, using a two pulse phase modulation (TPPM) decoupling scheme (39). Two dimensional (2D) 13C-13C correlation spectra were obtained using radio frequency-assisted diffusion (RAD) (40,41) with a mixing period of 25 ms.

NMR data processing— All 2D spectra were processed in NMRPipe (42) and visualized using CcpNmr Analysis (43). The number of points used for Fourier transformation was doubled by zero filling in each dimension. In some instances linear prediction was employed in the indirect dimension. An exponential line broadening function of 125 Hz was applied to each free induction decay (FID). Cα and Cβ chemical shift distributions for various secondary structures was obtained from Wang and Jardetzky (44).

RESULTS

Lysine point mutations induce β-sheet secondary structure in the crosslinking domains of EPs.

The control elastin-like polypeptide used in this study, designated EP20-24, consists of K-type crosslinking domains 21 and 23, flanked by hydrophobic domains 20 and 24 (Table 1). At approximately 10 kDa, EP20-24 is much smaller than tropoelastin, but retains important structural and functional properties of full-length
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tropoelastin, including the ability to coacervate (19,45). We have previously shown that this elastin-like polypeptide contains approximately 15% α-helix, as determined by CD spectroscopy at room temperature (17). Its reduced size and complexity makes it amenable for determining the effect of mutations on structure and function, as compared to full-length tropoelastin.

KA-type crosslinking domains are abundant and well-conserved in tropoelastins of most species (32-34). While lysine-derived crosslinks generated by the action of lysyl oxidase are also present in collagens, elastomeric proteins of non-vertebrates use other crosslinking mechanisms to stabilize their polymeric structures. For example, resilin, the highly efficient elastic protein of insect wing hinges, utilizes tyrosine residues to form a biphenyl crosslink called dityrosine (46,47). Since alignment of lysine side chains in tropoelastin and elastin-like polypeptides appears to take place through coacervation, we postulated that substitution of tyrosines for lysine residues in elastin-like polypeptides would result in a similar alignment of tyrosine side chains, permitting formation of dityrosine crosslinks. Similarly, stabilization of the polymeric form of elastomeric spider silks takes place through non-covalent β-sheet stacking of polyalanine sequences to create so-called liquid crystalline regions of the protein (48,49). We therefore investigated the possibility of generating these types of crosslinks in elastin-like polypeptides by modifying the crosslinking domains of EP20-24.

To this end, in one construct (designated EP:K to Y), all four lysine residues of EP20-24 were replaced with tyrosines. In a second construct (designated EP:K to A), all lysines were replaced with alanine residues. In both constructs the tyrosine following the second lysine in the crosslinking domain of EP20-24 was also mutated to alanine (See Table 1 for amino acid sequences).

The effects of these mutations on secondary structure of the polypeptides were monitored by far UV CD spectroscopy, using both EP20-24 and full-length human tropoelastin as controls. CD spectra observed for EP20-24 and tropoelastin at 5 °C were very similar, with strong minima at 222 nm and 202 nm (Fig. 1A and B). The minimum at 222 nm has previously been attributed to α-helical structure in crosslinking domains. The minimum at 202 nm has been characterized as the ‘disordered’ signal of the hydrophobic domains, and attributed to short polyproline II (PPII) backbone conformations and hydrogen bonded β-turns in the hydrophobic domains (4,50,51).

To determine the stability of secondary structures, the CD spectra of EP20-24 and full-length human tropoelastin were measured at increasing temperatures (Fig. 1A and B). Absorbance at 222 nm and 202 nm decreased with increasing temperature, indicating a loss in secondary structure in both the hydrophobic and crosslinking domains. Interestingly, absorbance at 222 nm had already essentially disappeared by 37 °C, signifying the loss of α-helical structure in both tropoelastin and EP20-24 at and above physiological temperatures. These findings indicate that, under physiological conditions, the α-helical structure in crosslinking domains of both the elastin-like polypeptides and full-length tropoelastin is extremely unstable.

The EP:K to A mutant displayed CD spectra similar to EP20-24 at 5, 25 and 40 °C, indicating α-helical and turn content similar to that of the native sequences. However, the absorbance signal at 202 nm was lost above 40 °C, and there was a shift to a strong minimum at 217 nm, indicative of β-strand formation (Fig. 1C). Furthermore, a precipitate formed in the sample chamber at 80 °C that was not observed during the melting of EP20-24 or tropoelastin. These results suggested temperature-induced aggregation of the polypeptides, coinciding with a secondary structure transition from α-helix and disorder to substantial β-strand formation. At 5 °C the CD spectra of EP:K to Y already showed a strong minimum at 217 nm with a very weak minimum at 202 nm, demonstrating that, even at low temperatures, this mutant polypeptide was predominantly β-strand in character, with no detectable α-helical structure (Fig. 1D). Heating of the sample to 37 °C resulted in loss of any trace of a minimum at 202 nm, indicating that the EP:K to Y polypeptide had an even greater propensity for β-strand formation than the EP:K to A mutant.

Mutant EPs form aggregates with spectroscopic characteristics of amyloid fibrils.

Observations of aggregation and increased content of β-strand secondary structure in the...
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mutated elastin-like polypeptides suggested the possible formation of the type of stacked β-sheet structures seen in amyloid fibrils. Binding of the fluorescent dye ThT has been used as a specific marker of the characteristic cross-β structures formed in amyloid fibril aggregates (52). A characteristic strong fluorescence signal at 482 nm was observed at increasing concentrations of the EP:K to Y mutant in the presence of ThT. Indeed, at equivalent molar concentrations, the amount of fluorescence surpassed that observed using the Aβ(1-28) amyloid peptide as a positive control (Fig. 2). Similarly, a solution of the EP:K to A, previously heated to 80 °C, also exhibited fluorescence emission at 482 nm in the presence of ThT, although the fluorescent yield was smaller. As expected, the control EP20-24 showed no detectable interaction with ThT, even at the highest concentrations used. These results demonstrate clearly that the amino acid replacements of the lysine residues in the crosslinking domains rendered the elastin polypeptides susceptible to ordered aggregation resulting in amyloid-like fibril formation.

Mutant elastin-like polypeptides have multiple possible aggregation pathways.

Amyloid fibril formation can be provoked by changes in solvent pH or polarity (53-55), and therefore may not reflect the ability to form such fibrils under normal physiological conditions. To ensure that the fibrils formed from mutant elastin-like polypeptides were not an artifact of the purification process (EP:K to Y), or a consequence only of heat-induced aggregation (EP:K to A), we assessed fibril formation under physiological conditions from an initial solution containing monomeric species. To disaggregate any preformed amyloid-like fibrils in the sample, the lyophilized EP:K to Y polypeptide was dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP), a fluorinated solvent known to solubilize amyloid-like aggregates. This highly volatile solvent was subsequently evaporated using a vacuum pump desiccator.

The HFIP-treated EP:K to Y, redissolved in 50 mM sodium phosphate, pH 7.0, exhibited a CD spectrum substantially similar to that of the EP20-24 control, with striking loss of the minimum at 217 nm and the addition of minima at 202 and 222 nm (Fig. 3A). This spectrum indicated that HFIP treatment abolished the β-sheet secondary structure seen previously (Fig. 1D), replacing it with a secondary structure more similar to the EP20-24 polypeptide, containing α-helical crosslinking and disordered hydrophobic domains. Furthermore, no fluorescence emission at 480 nm was observed at day 0 when ThT was added to HFIP-treated EP:K to Y that had been freshly dissolved in PBS, pH 7.4 (Fig. 3B). These results confirmed that any EP:K to Y amyloid-like aggregates were abolished by HFIP treatment, leaving a secondary structure very similar to that of the native polypeptide.

However, continued incubation of the HFIP-treated EP:K to Y in PBS at 37 °C in multiple microtiter plate wells resulted in the appearance of ThT fluorescence by day 2, with strong binding achieved after 3-4 days in three independent samples (Fig. 3B). Thus, despite being restored to an elastin-like monomeric secondary structure by HFIP, the EP:K to Y mutant had a strong predisposition to revert to β-sheet secondary structure and amyloid-like aggregation. In contrast, EP20-24 incubated for the same period of time showed no propensity for ThT binding, while only relatively weak ThT binding developed during incubation of EP:K to A (Fig. 3B). The presence of amyloid-like fibrils in the EP:K to Y samples was substantiated by TEM. Long fibrils, approximately 10 nm in diameter and appearing clumped and intertwined were observed by TEM (Fig. 3C). Fibrils were not observed in the coacervate of EP20-24.

CD spectra of both the EP:K to A polypeptide, prior to temperature-induced melting, and the EP:K to Y polypeptide, immediately after HFIP treatment, were similar to that of the EP20-24 control polypeptide, suggesting that all had elastin-like secondary structures. We therefore investigated the ability of these mutated polypeptides to undergo coacervation. Coacervation (liquid-liquid phase separation to form a suspension of colloidal droplets) is a prominent characteristic of elastin-like polypeptides. Coacervation profiles for EP20-24, EP:K to A and freshly dissolved HFIP-treated EP:K to Y polypeptides are shown in Figure 4A. All three polypeptides demonstrated classic coacervation profiles, with the rather surprising observation that both the EP:K to A polypeptide...
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and the EP:K to Y polypeptides showed an increased propensity for coacervation, indicated by lower coacervation temperatures than EP20-24.

We and others have previously shown that colloidal droplets in suspensions held above the coacervation temperature undergo a maturation process involving growth by coalescence and/or clustering (21,29,56,57). We therefore used light microscopy to compare maturation morphologies of the coacervate droplets formed by these three polypeptides. As expected, coacervate droplets of EP20-24 grew rapidly by coalescence, but remained as isolated spherules, even 60 minutes after initiation of coacervation (Fig. 4B). In contrast, droplets formed by both EP:K to A and EP:K to Y, were substantially smaller in size after 60 minutes, and exhibited marked clumping and assembly into beaded string-like morphologies (Fig. 4C and D). Such clumping behaviour of colloidal droplets has previously been reported by us (56), and is particularly characteristic of elastin-like polypeptides with increased tendencies for β-sheet formation (30).

Coacervation temperature correlates with hydrophobicity and β-sheet propensity.

In general, the coacervation temperature of elastin-like polypeptides has been shown to decrease with increased concentration and molecular weight, as well as with increased solution ionic strength (19,45,57). This phase separation can also be promoted by the addition of relatively low concentrations of TFE to the solution (17). Sequence and context of hydrophobic domains has also been demonstrated to affect coacervation temperature (17,30,45,58). However, most of these studies have only investigated alterations in hydrophobic sequences, and it is not clear whether mutations in crosslinking domains would have similar effects on coacervation. Since both the EP:K to Y and EP:K to A modifications to EP20-24 had the effect of lowering coacervation temperature (Fig. 4A), we undertook a systematic study of the effects of lysine substitutions on coacervation temperature, and correlated these effects with propensities for α-helical and β-strand secondary structure predicted for the position of the first lysine residue in the crosslinking domain (K1) (59). Substitutions of 1, 2, or 4 tyrosine residues for lysines in the crosslinking domain had increasing effects to lower the coacervation temperature, although the specific site of the substitution was not important (Table 1). In general, there was a strong correlation between increasing overall hydrophobicity (cumulative values from (60), relative to that of EP20-24 assigned a value of 0) and decreasing coacervation temperature (Fig. 5A, see also Table 1). Furthermore, a similar correlation was observed between predicted propensity for β-strand structure at the K1 location and decreased coacervation temperature (Fig. 5B). There was no discernible relationship between propensity for α-helical structure and coacervation temperature (Fig. 5C).

MAS NMR identifies conformational transitions between α-helix and β-strand in the crosslinking domains.

EP20-24 and EP:K to Y polypeptides were uniformly labeled with 13C and 15N, and characterized further by MAS solid-state NMR at 35 °C. Chemical shift assignments were generated by identifying amino acid-specific spin systems in 2D 13C-15C RAD correlation spectra. Isotropic chemical shift values of alanine and lysine were of specific interest because the crosslinking domains are largely comprised of these amino acids. Cα and Cβ chemical shift values are particularly sensitive to backbone torsion angles, making them ideal for identifying secondary structure components (44,61).

Alanine residues of lyophilized EP20-24 displayed Cβ-Cα cross peaks with chemical shift values indicative of a larger population consisting of α-helix and another smaller population of random coil (Fig. 6A). This experiment provides a good internal reference for optimal local structure content because internal hydrogen bonding in proteins is accentuated in the absence of water (62). As a result, it was expected that this sample would have a high proportion of α-helix. Remarkably, the hydrated coacervate of EP20-24 had no noticeable resonances indicating the presence of α-helix but displayed a conspicuous Cβ-Cα cross peak with shifts indicative of a β-strand backbone conformation (Fig. 6B). However, the hydrated EP20-24 polypeptide, crosslinked with genipin, showed a very prominent cross peak with chemical shift values indicative of alanines exclusively in an α-helical
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conformation (Fig. 6C). Hydrated fibrils prepared from the EP:K to Y polypeptide displayed Cβ and Cα chemical shifts corresponding to β-strand (Fig. 6D), as might be expected for amyloid-like fibrils formed by stacking of β-sheet crosslinking domains. Alanine cross peaks observed for hydrated coacervates of HFIP-treated EP:K to Y also showed exclusively β-sheet structure (Fig. 6E), similar to that observed for the coacervate of EP20-24 (Fig. 6B). Most Cβ-Cα cross peaks were broad, especially for the samples containing chemical shifts indicative of β-strand, suggesting a significant amount of backbone structural heterogeneity. The proline Cγ-Cδ cross peak observed in dry EP20-24 is not seen in the other spectra, since increased molecular motion of the hydrophobic domains of hydrated elastin inhibits cross-polarization to these spins.

These observations were supported by changes in the lysine Cβ-Cα chemical shifts of lyophilized, coacervated and crosslinked EP20-24 (Fig. 7). Two resonances corresponding to lysine in a β-sheet conformation were identified in the coacervate sample (Fig. 7A) but were noticeably absent in the crosslinked (Fig. 7B) and lyophilized samples (Fig. 7C). As for the spectra shown in Figure 6, broad cross peaks corresponding to proline resonances are observed in the lyophilized sample. These are absent in the presence of hydrophobic domain motion in the hydrated samples. The crosslinked sample had an additional cross peak (indicated by a dotted line) that may correspond to α-helical lysine residues, although it is partially overlapped with the valine Cβ-Cα crosspeak, preventing unambiguous assignment.

DISCUSSION

Assembly of the extracellular elastic matrix is a complex process, involving not only closely regulated synthesis of tropoelastin but also interactions with a number of other proteins as the tropoelastin is trafficked through the cell, secreted into the extracellular compartment, and incorporated into the growing elastic fibers. Because of the lack of normal turnover of these elastic fibers they must have the structural integrity, for example in the aorta, to withstand literally billions of cycles of stretch and recoil over a normal human lifespan without mechanical failure. Thus, assembly into the proper polymeric network architecture is critical for the physiological function and durability of elastic tissues.

While interactions with other matrix-associated proteins are clearly important for assembly, both full-length tropoelastin and also smaller polypeptides modeled on sequences and domain arrangements of tropoelastin have intrinsic properties of self-assembly, allowing them to spontaneously form network structures in vitro, even in the absence of other proteins. These networks can be crosslinked into polymeric materials with elastic properties similar to the native polymer. For this reason, proteins and polypeptides modeled on elastin sequences have been an important tool for exploring the relationship between the amino acid sequence, self-assembly and functional properties of elastin.

Coacervation has been recognized as the first step in elastin polymeric assembly both in vitro and in vivo, and many studies have demonstrated the influence of the 'disordered' or conformationally flexible hydrophobic domain sequences on this phase separation process. On the other hand, crosslinking domains, particularly the most abundant KA-type in which lysine residues destined for crosslinking are located in polyalanine sequences, have generally been considered to be relatively stable, passive participants in this assembly process, brought into alignment through interactions of the hydrophobic domains on phase separation. However, the data presented here clearly demonstrate that such KA-type crosslinking domains are much more structurally labile than previously appreciated, indicate that crosslinking domains are important for both regulating and mediating self-assembly of elastin polypeptides, and suggest that the presence of lysine side chains is necessary to prevent unwanted amyloid-like aggregation.

Both in the EP20-24 polypeptides, representing a native sequence in tropoelastin and in full-length tropoelastin itself, CD evidence for α-helical structure, although detectable at lower temperatures, was essentially absent at physiological temperatures. These observations are consistent with previous reports of limited actual as compared to predicted helix content of tropoelastin and elastin-like polypeptides containing crosslinking domains (17,20,63). Short,
isolated polyalanine sequences punctuated with lysine residues, such as those in crosslinking domains of elastin, have been described as poor α-helix formers (64-66). Furthermore, polyalanine sequences in other proteins have been reported to be structurally ambivalent, able to switch between random coil, α-helix or β-strand, depending on protein concentration, solution conditions, sequence context and length of the polyalanine sequence (67-76). Indeed, our data from solid-state NMR show clearly that, at physiological temperature, alanine and lysine residues in the crosslinking domains of coacervates of the native elastin-like polypeptide, EP20K24, are present almost exclusively in β-strand structures. Conversely, after formation of a polymeric network by covalent crosslinking of the lysine side chains using genipin, these residues are predominantly in α-helical conformation.

The specific structures formed by elastin in its various states and how they relate to assembly and function as an elastomer has been particularly contentious. This is largely due to the limitations of techniques such as CD, FTIR and x-ray crystallography to capture high resolution structural information on elastin, a protein that is both conformationally dynamic and largely unordered, even in its crosslinked, polymeric form.

Solid-state NMR has been used previously to provide information on the structure of insoluble elastin (9,77-79), but these investigations have generally focused on characterization of the mobile, hydrophobic domains of the protein and provided little or no insight into detailed structure of the crosslinking domains. Furthermore, no previous NMR study has addressed the question of the structure of elastin in the coacervated state.

The earliest suggestions of an α-helical structure for the crosslinking domains in elastin were based on the ability of this conformation to bring pairs of lysine residues separated by two or three alanines to the same side of the α-helix to allow the formation of the desmosine crosslinks, which involve the participation of four lysine side chains, presumably two from each crosslinking domain (18). However, direct evidence for the presence of α-helical structure in coacervated tropoelastin before crosslinking is very limited (80). Typically, it has been assumed that structures observed in solution or the dry state are preserved in the coacervate, and that these structural motifs may influence coacervation propensity. We and others have reported correlations between increased α-helical content and lower coacervation temperatures, which are usually taken as a measure of propensity for self-assembly (17,81). On the other hand, recent studies from our laboratory have shown that polypeptides with increased β-sheet content, as shown by FTIR, increase coacervate droplet stability (30). By systematically mutating lysines at the K1 position of EP20-24 to a wide range of amino acids with different side chain properties we have shown that there is a very strong correlation between the hydrophobicity of the crosslinking domains and the coacervation propensity of the polypeptide, independent of the secondary structure propensity of the substitution. This strongly suggests that the primary influence on coacervation tendency is the overall hydrophobicity of the elastin polypeptide and not the specific structures that may exist in solution. While we observed a reasonable correlation between β-sheet propensity of amino acid substitutions at the K1 position and coacervation propensity, it was weaker than that observed for the hydrophobicity of the substitution. This secondary correlation was likely observed because residues with high β-sheet propensity also tend to have hydrophobic side chains.

Although the reduction in coacervation temperature of elastin and elastin-like polypeptides by TFE has been attributed to stabilization of α-helical structure in crosslinking domains (81), the fact that a similar effect is seen on isolated hydrophobic domains that cannot form α-helical conformations (17) suggests that this effect of TFE may be due to more general modifications to the solvation properties of the solution.

Although we cannot comment on elastin structure immediately following the onset of coacervation due to our experiments being signal averaged over a period of multiple days, our solid-state NMR data clearly show β-strand structure dominates the crosslinking domains of a stable but uncrosslinked coacervate. Such β-strand structure is likely promoted by the crowded environment and lowered water content of the coacervate. In
Conformations of elastin crosslinking domains

general, as hydration levels decrease, lost peptide backbone-water hydrogen bonding is replaced with secondary structure formation (62). Indeed, in model polyalanine peptides increased protein concentration has been reported to result in a shift in conformational equilibrium to β-sheet structure (73,74). Molecular dynamics simulations of hydrophobic domain sequences of elastin have also demonstrated a decrease in backbone hydration accompanied by an increase in secondary structure in an aggregated state, although in this case hydrogen bonded β-turns predominate because of inhibition of the formation of β-sheet in these proline-rich domains (6). Although crosslinking domains are not directly responsible for phase separation, they must adapt to the reduced hydration levels presented by the coacervate environment. Our MAS NMR data indicate that they do this through the formation of β-sheet.

The data reported here provide important new insights into the conformational versatility of the crosslinking domains of elastin, and structural changes that may be taking place during the process of self-assembly and crosslinking. In solutions of tropoelastin prior to self-assembly it is clear that α-helical structure in the crosslinking domains is only stable at lower temperatures or in the presence of co-solvents such as TFE. At physiological temperatures, less ordered random coil structure appears to predominate in these domains. In the self-assembly process, as phase separation takes place, increased protein concentration and decreased hydration would promote a shift to secondary structure in the crosslinking domains. This could be satisfied by formation of hydrogen bonds stabilizing either α-helix or β-sheet, such that an equilibrium may exist between these two structures, at least initially after coacervation. Indeed, transient β-sheets formed in the coacervate may help to align and organize crosslinking domains for joining of lysine side chains. However, it appears that crosslinking domains in mature coacervates are predominantly in β-strand conformations, perhaps because of greater charge repulsions between closely spaced lysine side chains in an α-helical conformation. While β-strands predominate in mature coacervates, there is no evidence that these can go on to form stacked β-sheet, amyloid-like fibrillar structures binding ThT. However, replacement of crosslinking domain lysines with alamines or tyrosines results in the spontaneous formation of amyloid-like aggregates binding ThT, suggesting that the presence of the lysines in these domains is crucial to prevent the formation of such stable aggregates, likely through charge repulsion.

Finally, the MAS NMR data clearly demonstrate that, after crosslinking, these domains shift exclusively to α-helical structure. It is remarkable that this takes place even when crosslinking is mediated by genipin, which can only bridge between single lysine side chains, rather than by desmosine crosslinking, which has the added restraint of joining of lysine pairs sharing the same polypeptide backbone. Similar to disulfide linkages, a genipin crosslink between separate backbone chains will not restrict the conformation of one backbone to the phi-psi torsion angles of the other. That being said, single chain linkages between distant or separate backbone chains are known to have dramatic effects on secondary structure stability (82-84). In our case it is not clear whether this shift to α-helix is due to steric hindrance as a result of the crosslink formation, or is simply due to removal of lysine side chain charge repulsion. In any case, these data not only indicate previously unappreciated conformational transitions that may take place in crosslinking domains during assembly and polymerization of elastin, but also suggest that the presence of the charged lysine residues are important for preventing amyloid-like aggregation of these domains during self-assembly.
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FOOTNOTES

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3The abbreviations used are: TFE, trifluoroethanol; IPTG, isopropyl β-D-1 thiogalactopyranoside; ThT, Thioflavin-T; MAS, magic angle spinning; 2D, two dimensional; RAD, radio frequency-assisted diffusion; rf, radio frequency; TPPM, two pulse phase modulation; FID, free induction decay; PPII, polyproline II; HFIP, 1,1,1,3,3,3 hexafluoro-2-propanol; TEM, transmission electron microscopy

FIGURE LEGENDS

Figure 1. Effect of tyrosine and alanine substitutions on secondary structure of elastin-like polypeptides. CD spectra of elastin-like polypeptides at varying temperatures dissolved in 50 mM sodium phosphate buffer pH 7. A: EP20-24. B: human tropoelastin. C: EP:K to A. D: EP:K to Y.

Figure 2. Thioflavin-T (ThT) binding induced by mutations in crosslinking domains of the elastin polypeptide. Fluorescence emission of 10 mM ThT at 482 nm in the presence of varying concentrations of EP:K to Y (solid circles), Aβ1-28 (open circles), EP:K to A heated to 80 °C (solid triangles) and EP20-24 (open triangles).

Figure 3. Effect of HFIP treatment on the structure and aggregation properties of EP:K to Y. A: CD spectra of HFIP-treated EP:K to Y after evaporation of HFIP (dotted line), EP20-24 (solid line) and untreated EP:K to Y (dashed line) dissolved in 50 mM sodium phosphate buffer pH 7. B: Time course of amyloid formation as detected by ThT fluorescence at 480 nm. EP20-24 (solid line), EP:K to A (dotted line) and EP:K to Y treated with HFIP (dashed lines, 3 different wells denoted by (1), (2) and (3)) were dissolved in PBS pH 7.4 and incubated at 37 °C. C: Transmission electron microscopy of EP:K to Y after 3 days of incubation in PBS pH 7.4 at 37 °C.

Figure 4. Effect of tyrosine and alanine substitutions on coacervation characteristics of elastin-like polypeptides. A: Coacervation of EP20-24 (solid line), HFIP-treated EP:K to Y (dashed line) and EP:K to A (dotted line) dissolved in PBS pH 7.4 containing 1.5 M NaCl as monitored by absorbance at 440 nm upon increasing temperature at 1 °C/min. Bright field microscopy of elastin-like polypeptides dissolved in PBS pH 7.4 containing 1.0 or 1.5 M NaCl. B: EP20-24. C: EP:K to A; D: HFIP-treated EP:K to Y. Arrows in panels C and D indicate clumping of coacervate droplets. White scale bars correspond to 10 μm.

Figure 5. Relationship between amino acid sequence and coacervation temperature of elastin-like polypeptides. Temperature at initiation of coacervation (Tc) was plotted vs. relative hydrophobicity (A), β-sheet propensity (B) and α-helix propensity (C) of various elastin-polypeptides containing amino acid substitutions in the crosslinking domains (See Table 1 for full list of substitutions). Hydrophobicity and sheet propensity plots fit by least squares linear regression (solid lines) yielded r² values of 0.84 and 0.58, respectively.

Figure 6. Effect of aggregation state on alanine Cα and Cβ chemical shifts. The alanine Cβ-Cα cross peak regions of 2D 13C-13C correlation spectra, obtained using 25 ms RAD mixing, are shown. A:
Lyophilized EP20-24 (dry). B: Hydrated EP20-24 coacervate. C: Hydrated EP20-24 crosslinked with genipin. D: hydrated EP:K to Y fibrils. E: hydrated EP:K to Y coacervate. F: Reference figure describing the normal distributions of alanine $\mathrm{C}\beta$ and $\mathrm{C}\alpha$ chemical shifts in $\alpha$-helix ($\alpha$) or $\beta$-sheet ($\beta$) from solved structures (44). The mean chemical shifts for each secondary structure type are represented by the center of each cross peak. The additional cross peak at $\sim$25.5 and $\sim$48.5 ppm in the dry sample is due to proline resonances that are only prominent in the absence of water for this experiment.

**Figure 7.** Effect of aggregation state on lysine $\mathrm{C}\alpha$ and $\mathrm{C}\beta$ chemical shifts. The lysine $\mathrm{C}\beta$-$\mathrm{C}\alpha$ cross peak regions of 2D $^{13}\mathrm{C}$-$^{13}\mathrm{C}$ correlation spectra are shown. A: Hydrated EP20-24 coacervate. B: Hydrated EP20-24 crosslinked with genipin. C: Lyophilized EP20-24 (dry). D: Reference figure showing the normal distributions of lysine $\mathrm{C}\beta$ and $\mathrm{C}\alpha$ chemical shifts in $\alpha$-helix ($\alpha$) or $\beta$-sheet ($\beta$) from solved structures (44). The mean chemical shifts for each secondary structure type are represented by the center of each cross peak.
TABLE 1

Elastin polypeptide coacervation temperatures, hydrophobicities and secondary structure propensities

Coacervation temperature ($T_c$) of 50 µM polypeptide in PBS with final concentration of 1.5 M NaCl, pH 7.4

| Poly-peptide$^a$ | Crosslinking domains (21-23) sequence$^b$ | $T_c$ ($°C$) | Relative hydrophobicity$^c$ | β-sheet propensity$^d$ | α-helix propensity$^d$ |
|----------------|------------------------------------------|-------------|----------------------------|----------------------|-----------------------|
| EP20-24        | EAQAAAAAKAAKYGVGTPAAAKAAKAAQF            | 42          | 0                          | 0.74                 | 1.07                  |
| EP:K to Y      | EAQAAAAAYAAYAYAYAYYAAAYYAQF              | 12          | 60.1                       | -                    | -                     |
| EP:K to A      | EAQAAAAAAAAYAAYAYAYAYYAAAYYAQF           | 33.5        | 14.5                       | -                    | -                     |
| K1Y            | EAQAAAAAYAAYGVGTPAAAKAAKAAQF             | 28.5        | 18                         | 1.29                 | 0.61                  |
| K1F            | EAQAAAAAFAAKYGVGTPAAAKAAKAAQF            | 28          | 32.1                       | 1.28                 | 1.12                  |
| K1H            | EAQAAAAAHAAYGVGTPAAAKAAKAAQF             | 34.5        | 3.6                        | 0.71                 | 1.24                  |
| K1I            | EAQAAAAAIAYGVGTPAAAKAAKAAQF              | 31          | 24.8                       | 1.6                  | 1                     |
| K1L            | EAQAAAAALLAYGVGTPAAAKAAKAAQF             | 29          | 26.6                       | 1.22                 | 1.34                  |
| K1N            | EAQAAAAANAYGVGTPAAAKAAKAAQF              | 37.5        | 3                          | 0.65                 | 0.73                  |
| K1S            | EAQAAAAASAAKYGVGTPAAAKAAKAAQF            | 36          | 3.2                        | 0.72                 | 0.79                  |
| K1T            | EAQAAAAATAAKYGVGTPAAAKAAKAAQF            | 33.5        | 6.1                        | 1.2                  | 0.82                  |
| K1V            | EAQAAAATVAAYGVGTPAAAKAAKAAQF             | 31          | 17                         | 1.65                 | 1.14                  |
| K1E            | EAQAAAAAEAYGVGTPAAAKAAKAAQF              | 38          | 1.6                        | 0.26                 | 1.53                  |
| K2Y            | EAQAAAAAKAAKYGVGTPAAAKAAKAAQF            | 27.5        | 18                         | -                    | -                     |
| K3Y            | EAQAAAAAKAAKYGVGTPAAAYAAKAAQF            | 27.5        | 18                         | -                    | -                     |
| K4Y            | EAQAAAAAAYAYGVGTPAAAKAAKAAQF             | 28.5        | 18                         | -                    | -                     |
| K1Y K2Y        | EAQAAAAAYAAYGVGTPAAAKAAKAAQF             | 18.5        | 36                         | -                    | -                     |
| K1Y K4Y        | EAQAAAAAAYAYGVGTPAAAYAAKAAQF             | 20          | 36                         | -                    | -                     |
| K1E K4E        | EAQAAAAAKAAKYGVGTPAAAEAEAEAYAQF          | 46.5        | 3.2                        | -                    | -                     |

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$^a$ – Elastin polypeptides consist of domains 20-21-23-24 with corresponding amino acid sequences;
20: FPGFGVGVGIGPGVAGVPGVGVPVGVPGVGVPG
24: GLVPGVGVAPGVPGVAPGVPGVGLAPGVPGVAPGVPGVAPGVPGAIGP

$^b$ – Position of amino acid substitutions in bold

$^c$ – Cumulative hydrophobicity (60) of substitutions relative to hydrophobicity of EP20-24

$^d$ – Helix and sheet propensities (59) for single amino acid substitutions at position K1
Figure 1

Conformations of elastin crosslinking domains

A

B

C

D

\( \theta \) (deg cm\(^2\)/dmol)

wavelength (nm)

\( \theta \) (deg cm\(^2\)/dmol)

wavelength (nm)

\( \theta \) (deg cm\(^2\)/dmol)

wavelength (nm)

\( \theta \) (deg cm\(^2\)/dmol)

wavelength (nm)

5°

26°

40°

60°

5°

25°

37°

50°

5°

25°

40°

60°

80°

5°

25°

40°

60°

80°
Figure 2
Figure 3

A

\[ \theta \text{ (deg cm}^2\text{/dmol)} \]

\[ \text{wavelength (nm)} \]

B

\[ \text{Fluorescence (480 nm)} \]

\[ \text{Day} \]

C

Image
Figure 4

A

\[ \text{A (440 nm)} \]

\[ \text{T (°C)} \]

B

C

D
Figure 5

A

$T_c (\degree C)$ vs. Relative hydrophobicity

B

$T_c (\degree C)$ vs. Sheet propensity

C

$T_c (\degree C)$ vs. Helix propensity
Figure 6
Figure 7
Conformational Transitions of the Crosslinking Domains of Elastin During Self-assembly
Sean E. Reichheld, Lisa D. Muiznieks, Richard Stahl, Karen Simonetti, Simon Sharpe and Fred W. Keeley

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