Structural Determinants of Cross-linking and Hydrophobic Domains for Self-Assembly of Elastin-like Polypeptides†

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ABSTRACT: Elastin is a major structural protein found in large blood vessels, lung, ligaments, and skin, imparting the physical properties of extensibility and elastic recoil to these tissues. To achieve the required structural durability of the elastic matrix, the elastin monomer, tropoelastin, undergoes ordered assembly into a covalently cross-linked, fibrillar polymeric structure. Human tropoelastin consists of 34 exons coding for alternating hydrophobic and cross-linking domains. Using a series of well-defined recombinant polypeptides based on human elastin sequences mimicking native elastin, we have previously investigated the role of sequence and context of hydrophobic domains in elastin self-assembly. Here, we demonstrate that the structure of both cross-linking and hydrophobic domains have significant effects on the assembly of these polypeptides. Removing a putative flexible hinge region in the center of a cross-linking domain substantially increased the α-helical content and strongly promoted their self-aggregation. However, while trifluoroethanol (TFE) promoted and urea inhibited self-assembly of these polypeptides, these effects were not predominantly due to altered α-helicity of the polypeptides. Our results suggest that, while increased α helicity also favors this process, the major effect of TFE to promote organized self-assembly of elastin-like polypeptides is likely related to direct effects of this cosolvent on hydrophobic domains. Such simple elastin polypeptide models can provide an important tool for understanding the relationships between sequence, structure, and polymeric assembly of elastin.

Elastin is an extracellular matrix protein found in tissues such as the large blood vessels, lung parenchyma, elastic ligaments, and skin, where it confers the properties of extensibility and elastic recoil. Human tropoelastin, the 70-kDa monomeric form of elastin, consists of 34 exons (for general reviews of elastin biochemistry, see refs *1* and *2*). Most exons of elastin code for either hydrophobic or cross-linking domains, with these domains arranged in an alternating fashion in the protein. Hydrophobic domains are rich in nonpolar amino acids (e.g., glycine, proline, valine, and leucine) and include many short tandem repeat and quasi-repeat sequences. For example, the most prominent tandem repeat in human elastin is PGVGVA, which is repeated 7 times in exon 24. Limited experimental data as well as some computational modeling studies predict a highly flexible structure for these hydrophobic domains, with many transient β-turns and β-hairpins (*3*−*11*). In contrast, most cross-linking domains are rich in alanine and contain lysine residues usually positioned in KxxK or KxxxK spacings. These domains are predicted to form α-helical structures, placing such lysine pairs on the same side of the helix, consistent with the formation of intermolecular covalent cross-links involving two such pairs of lysines (*1*).

To achieve the necessary structural integrity of the elastic matrix, monomeric elastin undergoes ordered assembly into an extensive polymeric network. During this process lysine residues in the cross-linking domains must be juxtaposed for the formation of the covalent cross-links that stabilize the polymeric structure. Details of this assembly process are still not well-understood. While other nonelastin proteins likely play a role in the ordered assembly of polymeric elastin (*12*−*16*), we and others have shown that both tropoelastin and polypeptides modeled on elastin sequences on their own have an intrinsic ability for organized self-assembly that aligns lysine residues for cross-linking into polymeric structures with the elastomeric properties of native elastin (*17*−*23*).

Organized self-assembly of tropoelastin and polypeptides modeled on tropoelastin sequences appears to require a process of hydrophobically driven, temperature-induced phase separation or coacervation, during which a polypeptide-rich second phase separates from the bulk solution on elevation of the solution temperature (*5*, *18*, *24*−*26*). In recent years, insights into the sequence and structural characteristics of elastin that determine its propensity for self-assembly through coacervation, have been provided both by our laboratory (*27*, *28*) and by others (*14*, *29*−*32*). Several studies have used full-length tropoelastin and tropoelastin with specific domain deletions to investigate this process (*14*, *29*, *30*, *33*, *34*). Using a more reductionist approach,

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Table 1: Compositions and Characteristics of Recombinant Human Elastin Polypeptides[a]

polypeptide	composition[a]	molecular mass (Da)	coacervation temperature[b] (°C)	α-helical content	
				predicted (GOR IV) (%)	measured (CD, in water) (%)
EP20-24	20-21-23-24	10 010	40.0	24	15.4
EP20-24[23U]	20-21-23[U]-24	9 954	23.5	28	28.4
EP20-24-24	20-(21-23-24)$_2$	16 992	29.0	28	8.5
EP20-24-24[23U]	20-(21-23[U]-24)$_2$	16 880	12.5	33	32.8
EP20-24-24[21Y/A]	20-(21[Y/A]-23-24)$_2$	16 808	36.5	29	11.0

[a] Exon sequences are given below. Mutations from native elastin sequences are shown in boldface. Exon 20, FPGFGVGVGGIPGVAGVPGVGGVPGVGGVPGVGISP; Exon 21-23, EAQAAAAAKAAKYGVGTPAAAAAKAAAKAAQF; Exons 21-23[U], EAQAAAAAKAAKY**AAAAA**AAAAAKAAAKAAQF; Exons 21[Y/A]-23, EAQAAAAAKAAK**A**GVGTPAAAAAKAAAKAAQF; Exon 24, GLVPGVGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVGVAPGVGVAPAIGP. [b] Coacervation temperatures were determined under standard coditions as described in the Experimental Procedures.

Tamburro and his colleagues (*3*, *32*, *35*) have characterized synthetic peptides representing separate elastin domains coded for by individual exons and have provided evidence that the structure of such domains in isolation reflect their structure in the protein as a whole. Our laboratory has used recombinantly produced polypeptides containing both hydrophobic and cross-linking domains that model both the sequences and alternating domain structures of elastin (*27*, *28*). From all of these studies, it is evident that the number of hydrophobic domains, their specific sequences, and their context in alternation with cross-linking domains all influence propensity for self-assembly.

Less information is available on the influence of the cross-linking domains on the ability of elastin and elastin-like polypeptides to self-assemble. Weiss and his colleagues (*36*) have suggested that stabilization of the α-helical structure in cross-linking domains, achieved by the addition of trifluoroethanol (TFE)[1] to the solvent system, increased propensity for coacervation in full-length tropoelastin. Here, using mutations in sequence of cross-linking domains, we confirm the effect of the α-helical content on elastin self-assembly. However, we also demonstrate that the major effect of TFE to promote coacervation in these elastin-like polypeptides is not due to increased α-helical structure in cross-linking domains but rather is related to an independent mechanism of TFE to facilitate coacervation.

EXPERIMENTAL PROCEDURES

Plasmid Construction. All plasmids were constructed in a pGEX-2T expression vector (Amersham Biosciences) with human tropoelastin sequences. Schematic representations of the elastin polypeptides are shown in Table 1. Polypeptides contain hydrophobic domains (exons 20 and 24 of tropoelastin) and cross-linking domains (exons 21 and 23 of tropoelastin) or mutations of these exon sequences. Exon 22 is not included because it is spliced out in all of the tropoelastin cDNAs characterized to date. These polypeptides are named for the hydrophobic exons that they contain (see Table 1).

Plasmid construction of polypeptides EP20-24, EP20-24-24, and EP20-24-24[21Y/A] have been described elsewhere (*27*, *28*).

The following primers were used in plasmid construction for EP20-24[23U] and EP20-24-24[23U]: 5Ala#1, CAAGGCTGCCAAGTACGCAGCAGCTGCTGCCGCAGCTGCA; 5Ala#2, GCTGCGGCAGCAGCTGCTGCGTACTTGGCAGC; *Bam*HI-Ex20, CTGCTAGGGGGATCCATGTTTCCCGGCTTT; Ex24-*Apa*I, GCTTCGGGCCCAATCGCGGGAGCCAC; *Apa*I-Ex21, CGATTGGGCCCGAAGCTCAGGCAGCAGCTG; and Ex24-*Eco*RI, CTGCCTAGGGAATTCCTAAGGGCCAATCGCGGGAG.

PCR reactions were carried out using ProofStart DNA polymerase (Qiagen, Mississauga, Ontario, Canada). Template and primer concentrations and PCR conditions were used as suggested by the manufacturer. PCR products were purified using a QIAEX II gel-extraction kit (Qiagen), and ligation was performed using a Rapid DNA ligation kit (Fermentas, Burlington, Ontario, Canada).

EP20-24[23U] (Human Elastin Exons 20-21-23[G14A, V15A, G16A, T17A, P18A]-24). A five alanine substitution mutation, was introduced into exon 23 of the EP20-24 construct using a primer pair ligation strategy. Primers 5Ala#1 and 5Ala#2 were annealed to create a 36-bp fragment with overhangs compatible to restriction enzyme sites *Sty*I and *Pst*I, which are present in exon 23, flanking the target sequence to be mutated. The pGEX-2T vector containing EP20-24 was digested with the same two enzymes to release the native fragment. The presence of an additional *Pst*I site within the vector made it necessary to perform a three-fragment linear ligation to reassemble EP20-24[23U]. After heat inactivation at 65 °C and NaCl supplementation, the ligation mixture was digested with *Bam*HI and *Eco*RI. The 336-bp digested fragment consisting of exons 20-21-23[U]-24 was finally religated into *Bam*HI/*Eco*RI-digested pGEX-2T vector to generate the EP20-24[23U] construct.

EP20-24-24[23U] (Human Elastin Exons 20-21-23[G14A, V15A, G16A, T17A, P18A]-24-21-23[G14A, V15A, G16A, T17A, P18A]-24). Primers *Bam*HI-Ex20 and Ex24-*Apa*I, as well as *Apa*I-Ex21 and Ex24-*Eco*RI, were used with EP20-24[23U] as the template to generate a 380-bp fragment consisting of exons 20-21-23[U]-24 and a 280-bp fragment consisting of exons 21-23[U]-24, respectively. EP20-24-24-[23U] was generated by inserting the *Bam*HI/*Apa*I-digested 380-bp fragment and *Apa*I/*Eco*RI-digested 280-bp fragment into the *Bam*HI/*Eco*RI-treated pGEX-2T vector.

All oligonucleotide primers were synthesized, and construct sequences were confirmed using facilities provided by The Centre for Applied Genomics at The Hospital for Sick Children.

Polypeptide Expression and Purification. The procedure for elastin polypeptide expression and purification has been described previously (*27*, *28*). Briefly, DNA constructs were transformed into BL21 cells, and single colonies were

[1] Abbreviations: TFE, 2,2,2-trifluoroethanol; CD, circular dichroism.

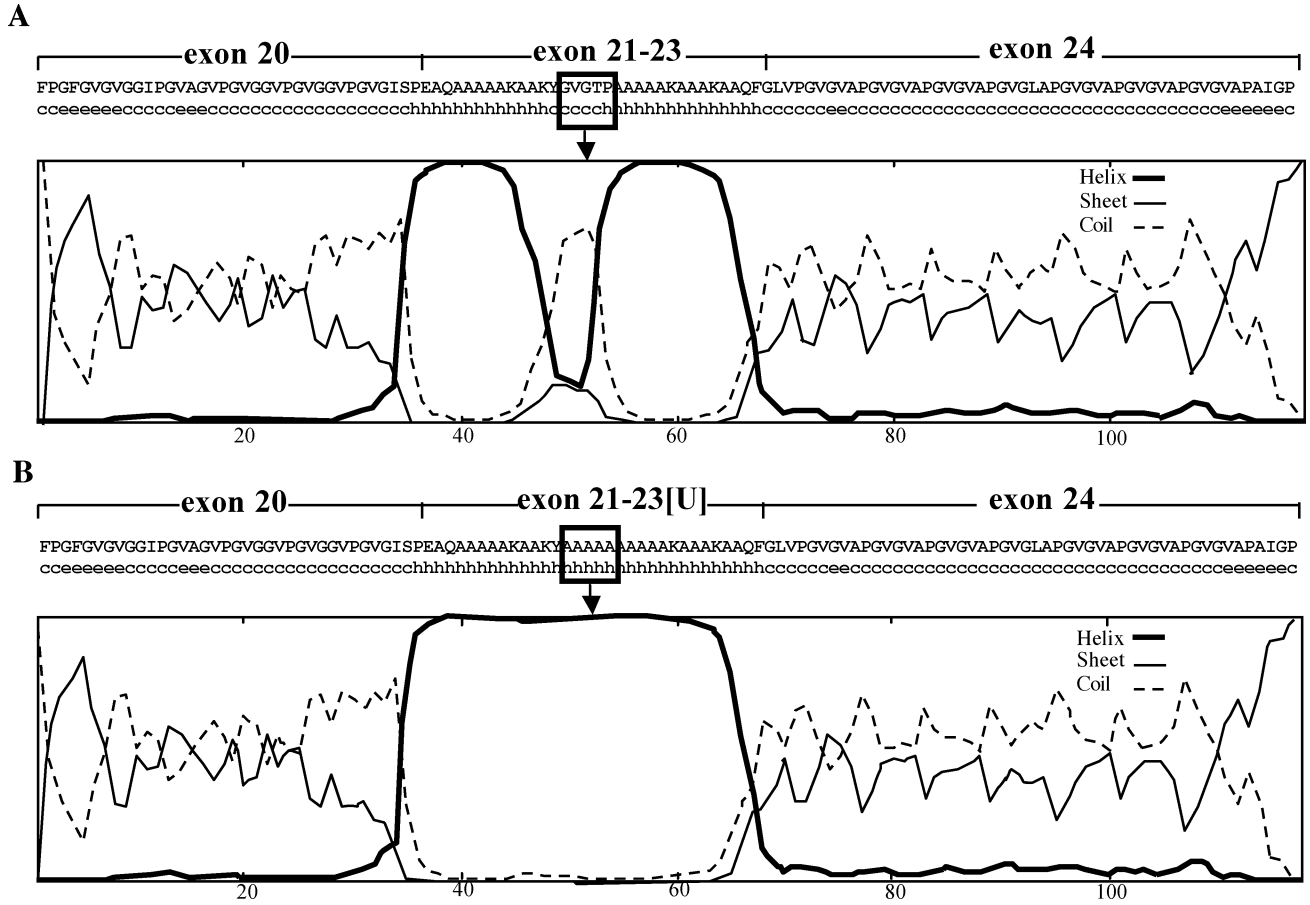

FIGURE 1: Predicted secondary structure of elastin polypeptides. Secondary structures of EP20-24 (A) and EP20-24[23U] (B) were predicted from amino acid sequences using GOR IV (http://au.expasy.org/).

inoculated in 2xYT with ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C overnight. This culture was then reamplified in 2xYT containing 2% glucose and ampicillin for 3.5 h (OD_{600} 0.8−1.0) before induction by adding isopropyl-α-D-thiogalactopyranoside (IPTG, 0.1 mM). The bacterial culture was harvested after another 4 h of incubation and digested with cyanogen bromide in 70% formic acid at room temperature overnight, followed by dialysis (3.5 K cutoff, Pierce, Rockford, IL) against water for 24−36 h. Elastin polypeptides were purified by Sephadex G25 (Amersham Biosciences) chromatography, eluting with 20 mM sodium acetate, followed by chromatography using a Sepharose SP (Amersham Biosciences) ion-exchange column eluted with 60 mM NaCl in 20 mM sodium acetate. Samples were then desalted on a Sephadex G25 column and subjected to reverse-phase HPLC using a Jupiter 10-μM C4 300 Å column (Phenomenex, Torrance, CA).

Amino acid compositions and concentrations of all polypeptides were determined by amino acid analysis, and molecular weights were confirmed by Q-TOF mass spectrometry using the facilities of the Advanced Protein Technology Centre, Hospital for Sick Children.

A synthetic polypeptide corresponding to exon 24 of human elastin was kindly provided by Dr. A. Tamburro, University of Basilicata, Italy.

Coacervation of Polypeptides. Standard coacervation conditions were 25 μM elastin polypeptide in 50 mM Tris buffer at pH 7.5, 1 mM $CaCl_2$, and 1.5 M NaCl. For experiments varying NaCl, TFE, and urea concentrations, polypeptides were dissolved in coacervation buffer (50 mM Tris at pH 7.5 and 1 mM $CaCl_2$) with NaCl, TFE (Sigma Chemical Co.), or urea (Invitrogen) to give the final concentrations as indicated. Coacervation was carried out by increasing the solution temperature at a rate of 1 °C/min and monitoring absorbance at 440 nm using a Cary 3 spectrophotometer equipped with a temperature controller. The coacervation temperature was determined as the temperature of the onset of increased absorption (*27*).

Circular Dichroism (CD) Spectrometry. CD spectra of human elastin polypeptides at 25 μM were obtained using an AVIV 62DS spectrometer at 4 °C. Polypeptides were first dissolved in water or coacervation buffer (50 mM Tris at pH 7.5 and 1 mM $CaCl_2$ final concentration), with NaCl and/or TFE added to give the final concentration as indicated. Protein concentrations for calculation of ellipticity were confirmed by amino acid analysis.

Western Blot Analysis. Elastin polypeptides were subjected to 15% SDS−polyacrylamide gel electrophoresis and immunoblotted using a polyclonal antibody raised against EP20-24 (*27*).

Determination of α-Helical Content. Predictions of α-helical content were based on the primary amino acid sequences of the polypeptides using GOR IV (*37*), available through the Expasy website (http://au.expasy.org/). The percentage of α-helix was determined by CD spectrometry using the ellipticity at a wavelength of 222 nm according to the formula of Chen et al. (*38*).

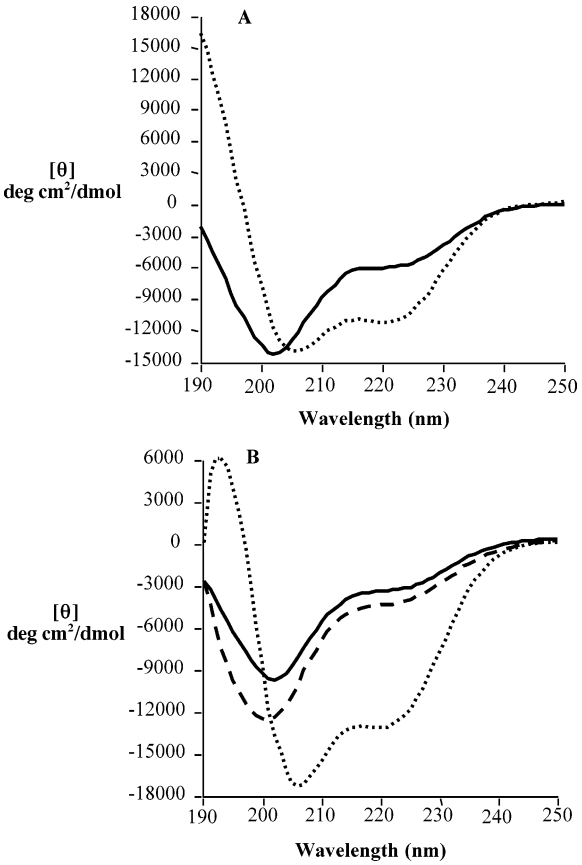

FIGURE 2: Effect on secondary structure of mutations in the cross-linking domains of elastin polypeptides. (A) CD spectra of EP20-24 (—) and EP20-24[23U] (•••) in water. (B) CD spectra of EP20-24-24 (—), EP20-24-24[21Y/A] (- - -), and EP20−24−24[23U] (• ••) in water.

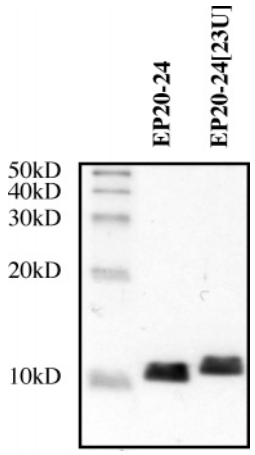

FIGURE 3: SDS polyacrylamide gel electrophoresis of EP20-24 and EP20-24[23U]. Polypeptides were separated on a 15% SDS−polyacrylamide gel and detected by Western blotting using a polyclonal antibody raised to EP20-24. The left lane contains standards with molecular weights as indicated.

RESULTS

Role of Cross-linking Domains in Determining the Structure and Coacervation of Elastin Polypeptides. Schematic representations of the polypeptides used, their molecular weights, and the amino acid sequences of individual exons are shown in Table 1. Polypeptides were named on the basis of their hydrophobic domains.

EP20-24 is the simplest elastin polypeptide in our model system, consisting of human elastin hydrophobic exons 20 and 24, flanking a single cross-linking domain consisting of human elastin exons 21 and 23. We had previously shown that this polypeptide, with a molecular weight of approximately 10 kDa, will undergo coacervation (*27*, *28*).

On the basis of its primary structure, GOR IV predicts that polypeptide EP20-24 will contain approximately 24% α-helix (Table 1), with that α-helical structure localized to the cross-linking domain (Figure 1A). This assignment of predicted structure is not surprising, because, of the 32 amino acids included in the cross-linking domain consisting of exons 21 and 23, 18 are alanine residues, which are known to promote the formation of α-helical structure in proteins (*39*). However, experimental measurements of α-helical content using CD indicate an α-helical content of only approximately 15% (Table 1 and Figure 2A), suggesting that the α-helical structure is imperfect or frayed, as might be expected for relatively short polyalanine sequences. Notably, GOR IV also predicted the presence of a non-α-helical region in the center of the cross-linking domain, which has the potential to function as a flexible "hinge" region in this structure, interrupting the α-helix (Figure 1A).

To increase the continuity of the α-helical region in EP20-24, the amino acid residues making up this hinge region (GVGTP) were mutated to alanine residues. This "unhinged" version of EP20-24 was designated EP20-24[23U]. As expected, GOR IV predictions based on the sequence of EP20-24[23U] indicated an increase in the α-helical content to 28% (Table 1) and the presence of an uninterrupted α-helix in the cross-linking domain (Figure 1B). CD measurements (Figure 2A) both confirmed an increase in the α-helical content of the mutated polypeptide and showed agreement between predicted (28%) and measured (28.4%) α-helical contents, indicating a considerable increase in the stability of the α-helical structure in this domain. Increased continuity and stability of the α-helical structure in the cross-linking domain would predict that the "unhinged" polypeptide might be more rodlike in structure. Western blot analysis using the antibody raised against EP20-24 (Figure 3) showed an increased apparent molecular mass for EP20-24[23U] as compared to EP20-24 (see Table 1 for actual molecular masses), consistent with a more stable rodlike structure.

Removal of the "hinge" region of EP20-24 with a consequent increase in α-helical content also had a substantial effect on coacervation of the polypeptide, decreasing the coacervation temperature under standard conditions from 40 to 23.5 °C (Figure 4A). Similar results were seen when both cross-linking domains in EP20-24-24 were mutated to the "unhinged" sequences. For EP20-24-24[23U], predicted and measured α-helical contents were in agreement at 33% (Table 1 and Figure 2B), and the coacervation temperature of this polypeptide was lowered to 12.5 °C compared to 29 °C for EP20-24-24 (Figure 4B).

These results were consistent with the data of Weiss and his colleagues (*36*) who correlated increased measured α-helical content of tropoelastin, induced by TFE as a cosolvent in the coacervation buffer, with decreased coacervation temperatures.

However, for our polypeptides, modifications to cross-linking domains do not in every case correlate α-helical content with coacervation temperature. EP20-24-24[21Y/A] is a polypeptide in which the single tyrosine residue in each

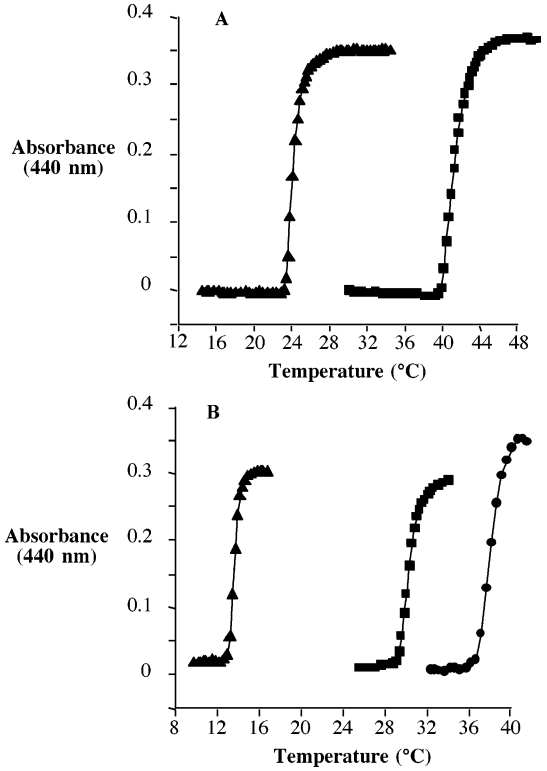

FIGURE 4: Coacervation characteristics of elastin polypeptides. (A) Coacervation (temperature-induced phase separation) of EP20-24 (■) and EP20-24[23U] (▲). (B) Coacervation (temperature-induced phase separation) of EP20-24-24 (■), EP20-24-24[21Y/A] (●), and EP20-24-24[23U] (▲). Coacervation buffer was 50 mM Tris at pH 7.5 containing 1 mM $CaCl_2$ and 1.5 M NaCl. Coacervation was followed by turbidity, measured by absorption at 440 nm.

of the two cross-linking domains has been mutated to an alanine. This mutation results in little or no change in predicted α-helical content (Table 1) and a small increase in measured α-helical content (Figure 2B), but the effect was to raise the coacervation temperature by approximately 7 °C (Figure 4B).

Effect of TFE on Coacervation of Elastin Polypeptides. Using CD measurements, Weiss and his colleagues (*36*) had shown that the presence of TFE as a cosolvent increased the α-helical content of tropoelastin, presumably by stabilizing α-helical structures in the cross-linking domains. The effect of TFE on the measured α-helical content of the elastin polypeptides EP20-24 and EP20-24[23U] in water is shown in Figure 5. Increasing the percentage of TFE from 0 to 50% raised the measured α-helical content of EP20-24 from 15 to 29%, as indicated by the deepening trough at 222 nm in the CD spectra (Figure 5A), suggesting a stabilization of the α-helical structure in the cross-linking domain. However, for EP20-24[23U], in which the α-helical structure of the cross-linking domain had already been stabilized by the mutation of the hinge region, TFE had little or no effect to further increase α-helical content (Figure 5B and 5C).

If decreased coacervation temperatures in the presence of TFE were primarily related to stabilization of α-helical structures in the cross-linking domains, it would be expected that TFE would have little or no effect to further decrease the coacervation temperature of EP20-24[23U], because the α-helical content of this polypeptide had already been maximized by the mutation eliminating the hinge region. To directly compare effects of TFE on α-helical contents and

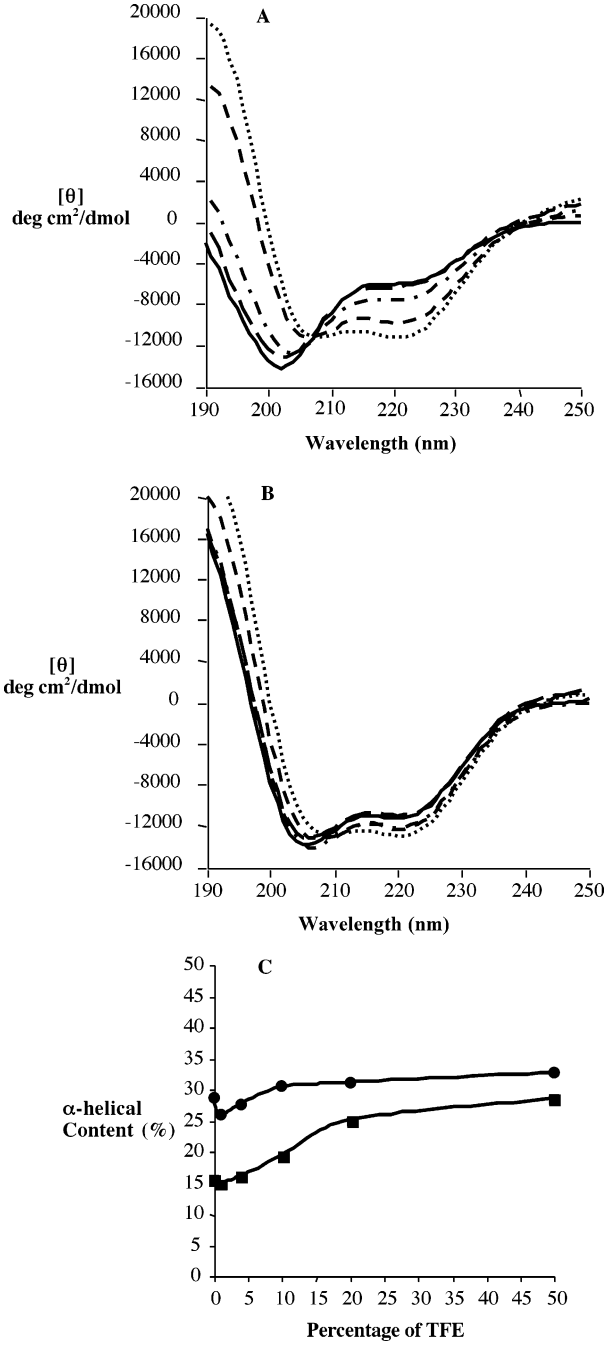

FIGURE 5: Effect of TFE on the secondary structure of elastin polypeptides. CD spectra of EP20-24 (A) and EP20-24[23U] (B) in water with 0% (—), 4% (— —), 10% (-·-), 20% (- - -), and 50% (···) TFE. (C) Relationship between the TFE concentration and α-helical content determined by CD spectrometry for EP20-24 (■) and EP20-24[23U](●).

coacervation temperatures for these polypeptides, it was necessary to use solution conditions suitable for coacervation. Therefore, 25 μM EP20-24 and EP20-24[23U] was prepared in coacervation buffer (50 mM Tris at pH 7.5, 1 mM $CaCl_2$, and 0.8 M NaCl) with varied amounts of TFE, and these solutions were used for both CD spectroscopy and coacervation experiments.

The effects of TFE on α-helical contents and coacervation temperatures of EP20-24 and EP20-24[23U] are shown in Figure 6. In these experiments, TFE concentrations were limited to a maximum of 7−10% by their effect on

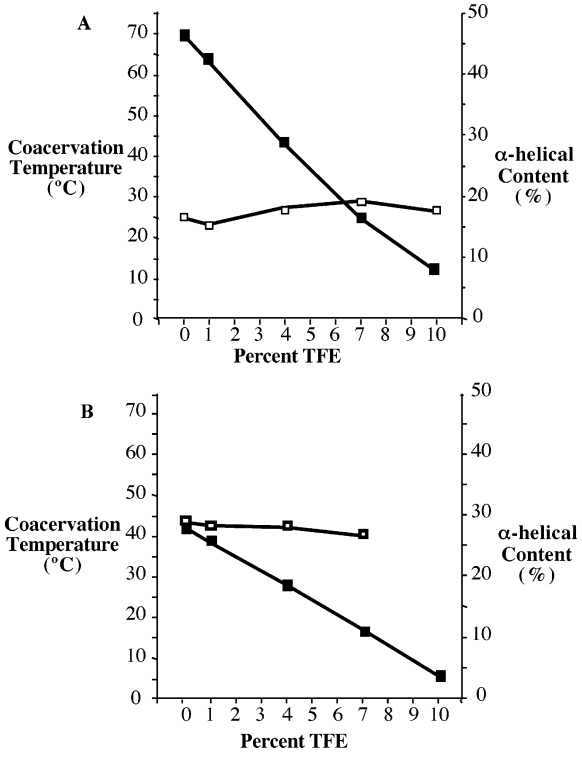

FIGURE 6: Effect of TFE on the α-helical content and coacervation temperature of elastin polypeptides. Relationship between the α-helical content determined by CD spectrometry (□) and coacervation temperatures (■) of EP20-24 (A) and EP20-24[23U] (B) in the presence of varied concentrations of TFE.

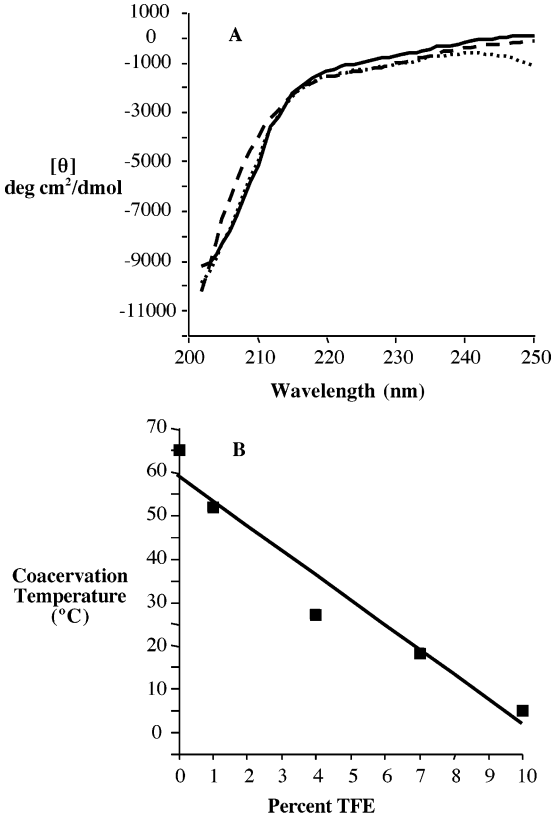

FIGURE 7: Effect of TFE on the secondary structure and coacervation temperature of elastin polypeptide corresponding to exon 24. (A) CD spectra of exon 24 polypeptide in water with 0% (—), 4% (- - -), and 10% (···) TFE. (B) Relationship between the TFE concentration and coacervation temperature of the exon 24 polypeptide. The exon 24 polypeptide (400 μM) was coacervated in 50 mM Tris buffer at pH 7.5 containing 1 mM $CaCl_2$ and 1.5 M NaCl with TFE concentrations as indicated.

coacervation temperatures. Concentrations of TFE between 0 and 10% had little effect on the α-helical content of EP20-24 in the coacervation buffer, remaining between 15 and 20%. However, coacervation temperatures fell from approximately 70 to 12 °C over this range of TFE concentrations (Figure 6A). Similarly, while concentrations of 0−7% TFE had essentially no effect on the α-helical content of EP20-24[23U] (approximately 28%), the coacervation temperature for this polypeptide was decreased by more than 20 °C (Figure 6B). These results suggest that the effect of TFE on the coacervation temperature was not primarily due to increased α-helical structure in the elastin polypeptides.

The effect of TFE on the coacervation temperature was further investigated using a polypeptide corresponding to exon 24, a hydrophobic domain of human elastin. Hydrophobic domains are generally believed to have conformational flexibility favoring β-turn and β-hairpin structures (*3*−*11*). The exon 24 polypeptide includes a 7-fold tandem repeat of PGVGVA, contains only nonpolar amino acids (Table 1), and is predicted by GOR IV to have no α-helical content (Figure 1A). The absence of the α-helical structure and the inability of TFE at concentrations of 0−10% to induce the α-helical structure in this polypeptide were confirmed by CD measurements (Figure 7A). Coacervation of the exon 24 polypeptide can be induced, although higher polypeptide concentrations and increased concentrations of NaCl in the coacervation buffer are required. The exon 24 polypeptide was prepared at a concentration of 400 μM in coacervation buffer (50 mM Tris at pH 7.5, 1 mM $CaCl_2$, and 1.5 M NaCl), with a varied percentage of TFE (0−10%). While these concentrations of TFE had no effect to induce the α-helical structure in this polypeptide, coacervation temperatures were lowered by approximately 60 °C (Figure 7B). These results were consistent with recently published observations by Pepe et al. (*35*), demonstrating that coacervation temperatures of peptides corresponding to sequences of proline-rich hydrophobic exons of tropoelastin (exons 18, 20, 24, and 26) were substantially decreased in the presence of 7.5% TFE, confirming that the effect of TFE on promoting the propensity for coacervation of elastin polypeptides is not primarily due to an increase in α-helical content of these polypeptides.

Effect of Urea on Coacervation of Elastin Polypeptides. While TFE is commonly used to stabilize α-helical structure in proteins, urea is a reagent known to cause protein unfolding by destabilizing the α-helical structure (*40*). We therefore investigated the effect of urea on coacervation temperatures of EP20-24 and the exon 24 polypeptide (Figure 8). For both polypeptides, urea at concentrations of 0−1 M did not alter the general shape of the coacervation curves (data not shown) but raised the coacervation temperature of both polypeptides by approximately 20 °C. While the effect of urea on EP20-24 might be related to some destabilization of the α-helical structure, the similar effect of urea on the exon 24 polypeptide, which contains no α-helix, again suggests that the α-helical content of these polypeptides is not the major determinant of their propensity for self-assembly.

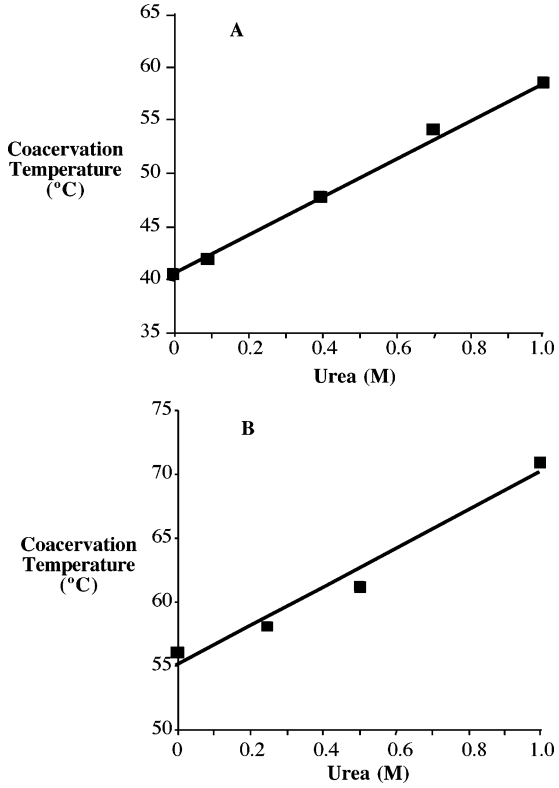

FIGURE 8: Effect of urea on the coacervation temperature of elastin polypeptides. Relationship between the coacervation temperatures and urea concentrations for EP20-24 (A) and the exon 24 polypeptide (B). EP20-24 (25 μM) and the exon 24 polypeptide (400 μM) were coacervated in 50 mM Tris buffer at pH 7.5 containing 1 mM $CaCl_2$ and 1.5 M NaCl with urea concentrations as indicated.

DISCUSSION

Assembly of polymeric elastin into the extracellular matrix *in vivo* is a complex process, likely involving several matrix proteins including fibrillins, fibulins, the elastin-binding protein, and lysyl oxidase, to achieve the proper architecture and extraordinary mechanical durability of this natural biomaterial (*12*−*16*). However, in recent years, it has become clear that tropoelastin, the monomeric form of elastin, itself contains sufficient structural information to allow self-assembly into an organized polymer. During this process of self-assembly, lysine side chains are juxtaposed, allowing the formation of lysine-derived covalent cross-links similar or identical to those present in native polymeric elastin (*19*−*22*). This propensity for organized self-assembly is also seen in polypeptides mimicking sequences and domain structures of elastin (*17*, *18*, *21*, *22*, *35*). Moreover, whether produced from full-length tropoelastin (*23*) or elastin-like polypeptides (*22*), the cross-linked material demonstrates elastomeric properties remarkably similar to those of native polymeric elastin.

Organized self-assembly of tropoelastin or elastin-like polypeptides takes place through a process of temperature-induced phase separation (coacervation) involving the formation of a protein-rich second phase. While this process has for the most part been studied *in vitro*, there have been suggestions that a coacervation-like assembly step is also important for *in vivo* formation of the elastin polymer (*17*, *41*, *42*). For this reason, our laboratory and others are investigating the relationship between polypeptide sequence and domain structure and propensity for self-assembly, as measured by the coacervation temperature. These studies have demonstrated that the number, sequence, and arrangement of hydrophobic domains all affect self-assembly (*27*, *28*). On the other hand, the role of cross-linking domain structures in this process has not been as closely investigated.

Cross-linking domains in tropoelastin are generally believed to be predominantly α-helical in structure. However, several laboratories have observed that the α-helical content of the protein, measured by CD, is substantially less than expected from the primary sequence prediction (*33*, *43*), suggesting the presence of less stable, frayed structures. Recently, Weiss and his colleagues (*36*) reported that stabilizing α-helical structures in full-length tropoelastin, achieved through the use of TFE as a cosolvent, not only raised the measured α-helical content but also significantly lowered the coacervation temperature of tropoelastin, leading to a proposed model in which the formation of α-helix assists the alignment of monomers and the interaction between hydrophobic domains, subsequently promoting the process of self-assembly.

Our data presented here using simple polypeptides modeled after domains of human elastin and containing both hydrophobic and cross-linking domains are, in the first instance, in agreement with Weiss's model (*36*); that is, stabilization of the α-helical structure in the single cross-linking domain of EP20-24 by mutations of the amino acids in the putative "hinge" region to alanines not only raised measured α-helical content to predicted values but also significantly lowered the coacervation temperature of the polypeptide. However, the role of TFE to promote self-assembly clearly involves more than simple stabilization of α-helical structures in these polypeptides, because a major effect of TFE on the coacervation temperature was also seen for polypeptides in which the α-helical content was either unaltered or not present at all. In this regard, Tamburro and his colleagues (*35*) have recently demonstrated that peptides corresponding to several proline-rich hydrophobic exons in human tropoelastin, including the same exon 24 sequence as reported here, showed a significant reduction in the coacervation temperature in the presence of 7.5% TFE. Our results using larger elastin-like polypeptides, which include both hydrophobic and cross-linking domains, are entirely in agreement with the data reported by Pepe et al. (*35*) for individual hydrophobic domains.

The structures of hydrophobic domains in tropoelastin and elastin-like polypeptides are of considerable interest because of their proposed roles both for the capacity of these proteins for organized self-assembly into polymeric structures and for the elastomeric properties of polymeric materials derived from these proteins. Most models, derived from both experimental and computational approaches, suggest that these domains are highly flexible in structure. Tamburro and his colleagues (*2*, *3*, *7*, *8*, *32*, *35*, *44*) have extensively studied structural characteristics of peptides corresponding to individual exons of human elastin. In general, for the proline-rich hydrophobic exons, they have reported a predominance of polyproline II-like structures in aqueous solution, with β-turn and unordered structures gaining increased prominence in organic solvents such as TFE (*3*, *35*). In addition, they showed that ionic strength, peptide concentration, and increased solution temperature, all factors which promote

coacervation, resulted in an increased proportion of more folded conformations, including turns (*35*).

This correlation of decreased polyproline II structure and increased folded conformations with conditions promoting coacervation might suggest that such folded conformations are important for the process of organized self-assembly into oligomers, which takes place as a result of coacervation. Consistent with this correlation, our data show that low concentrations of urea have a significant effect to raise the coacervation temperature in elastin polypeptides, which include both hydrophobic and cross-linking domains. The fact that concentrations of urea required to substantially destabilize even relatively short α-helical segments in proteins are considerably higher than those used here to affect coacervation of elastin-like polypeptides (*40*), together with the similar effect of urea on the polypeptide corresponding to exon 24, which contains no α-helical structure, clearly indicates that the effect of urea is unlikely due to destabilization of α-helical structures. A more likely explanation may be related to the observation of Bochicchio et al. (*32*) of increased stabilization of polyproline II structures in hydrophobic domains of elastin by urea.

The mechanism for the remarkable effects of TFE to lower the coacervation temperature of elastin-like polypeptides is not clear. Both our data presented here and that of Tamburro and his colleagues (*35*) demonstrate that this must largely be due to effects on hydrophobic domains. While it might be tempting to speculate that TFE has a direct effect on the structure of these hydrophobic domains, both our results on polypeptides containing both hydrophobic and cross-linking domains and those of Pepe et al. (*35*) on proline-rich hydrophobic exons show that large decreases in the coacervation temperature in polypeptides with or without cross-linking domains are not accompanied by significant changes in the secondary structure as detected by CD measurements. Either CD measurements are insufficiently sensitive to detect subtle shifts in conformer distributions, which have major consequences for coacervation behavior, or, as has been suggested by Tamburro and his colleagues (*35*), at these relatively low concentrations, TFE is acting as an "osmolyte", directly altering the interactions between the polypeptides and water (*45*−*48*), resulting in favoring hydrophobic interactions and the promotion of the process of coacervation.

In summary, our data obtained using mutations to the putative "hinge" region of the elastin-like polypeptide EP20-24 provide unambiguous confirmation of a relationship between the stability of α-helical structures in cross-linking domains and propensity for self-assembly. As suggested by Weiss and his colleagues, this may be related to an increased ability of more rigid, rodlike structures to form the fibrillar arrays seen in coacervates of elastin (*36*). At the same time, our data on elastin-like polypeptides containing both cross-linking and hydrophobic domains clearly demonstrate that the major effects of TFE to lower the coacervation temperature are not due to the increased α-helicity of the cross-linking domains in these polypeptides but rather, as reported by Pepe et al. (*35*), are related to effects of TFE on hydrophobic domains of these polypeptides. Details of the mechanism of this effect of TFE on the initiation of self-assembly and on the maturation of elastin-like polypeptides into ordered polymeric structures are currently under investigation.

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