Cooperativity between the Hydrophobic and Cross-linking Domains of Elastin*§

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The principal protein component of the elastic fiber found in elastic tissues is elastin, an amorphous, cross-linked biopolymer that is assembled from a high molecular weight monomer. The hydrophobic and cross-linking domains of elastin have been considered separate and independent, such that changes to one region are not thought to affect the other. However, results from these solid-state $^{13}$C NMR experiments demonstrate that cooperativity in protein folding exists between the two domain types. The sequence of the EP20-24-24 polypeptide has three hydrophobic sequences from exons 20 and 24 of the soluble monomer tropoelastin, interspersed with cross-linking domains constructed from exons 21 and 23. In the middle of each cross-linking domain is a “hinge” sequence. When this pentapeptide is replaced with alanines, as in EP20-24-24[23U], its properties are changed. In addition to the expected increase in $\alpha$-helical content and the resulting increase in rigidity of the cross-linking domains, changes to the organization of the hydrophobic regions are also observed. Using one-dimensional CPMAS (cross-polarization with magic angle spinning) techniques, including spectral editing and relaxation measurements, evidence for a change in dynamics to both domain types is observed. Furthermore, it is likely that the methyl groups of the leucines of the hydrophobic domains are also affected by the substitution to the hinge region of the cross-linking sequences. This cooperativity between the two domain types brings new questions to the phenomenon of coacervation in elastin polypeptides and strongly suggests that functional models for the protein must include a role for the cross-linking regions.

Elasticity in blood vessels and skin originates from elastin, an insoluble and amorphous protein assembled from its soluble monomer tropoelastin (1–3). Tropoelastin and insoluble elastin are typically described as having two types of domains, cross-linking and hydrophobic. The former are usually polyalanine regions, typically found as KAAK or KAAAK motifs, whereas the latter are dominated by polypenta- or polypentapeptide repeats. Because the molecular weight of tropoelastin is typically large (>70 kDa) and its composition complex, significant effort has been placed into identifying and characterizing elastin peptides (4–11). These mimetics range from the simple repeating polypeptides based on the VPGVG subunit, to those that more closely mirror the more complex native sequence. In particular, Keeley and coworkers (12–14) have recently reported a series of related polypeptides that are composed of alternating hydrophobic and cross-linking domains. These polypeptides have been shown to mimic various characteristics of the native protein, including coacervation and elasticity (12–15). Many structural questions may be addressed with these mimetics. For instance, does a change in the sequence of one domain type impact the other? Which modifications impact the overall protein structure? Moreover, how can these structural changes be used to identify key features of the functional models of the proteins?

For this study, two related elastin-like polypeptides were synthesized. EP20-24-24 has five domains, constructed from exons 20 and 24 for the hydrophobic, and 21/23 for the cross-linking, as follows: Ex 20, FPGFGVGGVPVAGVPGVGGVPGVGIS; Ex 21/23, PEAAAAAAKAKKYGVGTTAAAAKAAKAAQF; Ex 24, GLVPGVGAPGVGLAPGVYGAPGVGAPGVPGAVAIIAG; Ex 21/23, PEAAAAAAKAKKYGVGTTAAAAKAAKAAQF; and Ex 24, GLVPGVGAPGVGLAPGVYGAPGVGAPGVPGAVAIIAG.

Because both tropoelastin and mature elastin typically have long multidomain sequences of alternating hydrophobic and cross-linking domains very similar to EP20-24-24, this polypeptide (and longer ones, such as EP20-244) is a reasonable mimic. Experiments by Keeley and coworkers, again, show that many of the physical properties of these polypeptides closely mirror those of the native protein (12–15).

Another polypeptide was synthesized to be identical to EP20-24-24, except the central pentapeptidyl turn, or “hinge” (underlined in the above representation), in each of the cross-linking domains has been substituted by alanines. Hence, it is called the “unhinged” elastin polypeptide EP20-24-24[23U]: Ex 20, FPGFGVGGIPVAGVPGVGGVPGVGIS; Ex 21/23, PEAAAAAAKAKKYGVGTTAAAAKAAKAAQF; Ex 24, PEAAAAAAKAKKYGVGTTAAAAKAAKAAQF.

The abbreviations used are: EP20-24-24, elastin peptide with the sequence encoded by exons 20-21/23-24; EP20-24-24[23U], the unhinged elastin polypeptide; CP, cross-polarization; CPD, cross-polarization with depolarization; CPMAS, cross-polarization with magic angle spinning.
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GLVPGVGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVAPGV-
APGVGVAPAIG; Ex 21/23, PEAQAAAAAASKYAAAA-
AAAAKAAKAAAKQF; and Ex 24, GLVPGVGVAPGVGVAP-
GVAPGVGLAPGVGVAPGVAPGVAPGVAPAIG.

Table 1 illustrates the amino acid composition of EP20-24-24 and EP20-24-24[23U]. In addition to the obvious sequence similarities, the makeup of EP20-24-24 and EP20-24-24[23U] greatly resembles that of native elastin, with its predominance of the four small, hydrophobic amino acids: glycine, valine, proline, and alanine. Keeley and coworkers (14) have provided extensive evidence for changes in structure upon substitution of the hinge region by the five alanines. An increase in α-helical content from 8.5% in EP20-24-24 to 32.8% in EP20-24-24[23U] was determined by circular dichroism. In addition, the SDS-PAGE results of analogous hinged and unhinged elastin polypeptides show that the apparent molecular weight of the latter is higher, which is expected for a more rigid and rod-like structure. The SDS-PAGE results also indicate that the peptides are well behaved monomers. Finally, another reported result included the coacervation temperature of the unhinged polypeptide (12.5 °C), which is significantly lower than that observed for EP20-24-24 (29.0 °C).

Solid-state NMR spectroscopy is an ideal structural tool for the characterization of elastin and its related peptides. The amorphous nature of native elastin and its inaccessibility to other high resolution structural methods make it (and its related peptides) an ideal candidate for characterization by solid-state NMR. Numerous solid-state NMR studies of native and native-like elastin, as well as elastin polypeptides, have been reported (16–24), showing the power and versatility of the general approach. Overall, the current picture reflects that elastin is likely composed of a “conformational ensemble” in the hydrophobic domains with largely α-helical cross-linking regions. In addition, there are some unusual dynamics, particularly in the “liquid-like” nature of segments of the protein (16, 20, 22, 25).

As with the general collection of biochemical and biophysical work on elastin, previously reported NMR-based studies have focused on either the amorphous native protein, which is assembled from the high molecular weight monomer, or the repeating polypeptides based on hydrophobic sequences found in both tropoelastin and insoluble elastin. As noted earlier, although clearly interesting and relevant, there are some experimental shortcomings to using the native protein or the hydrophobic mimetic, particularly regarding complexity or lack thereof, respectively. However, the recombinant polypeptides described extensively by Keeley and coworkers appear to be a relevant and very compelling “happy medium,” because they preserve the alternating nature of native hydrophobic and cross-linking domains while keeping the polypeptides small enough to clarify interpretation. Furthermore, the use of a bacterial host allows future experiments to utilize strategic labeling for incorporation of stable isotopes, a necessary element of sophisticated structural measurements by NMR.

To characterize these two polypeptides, a number of one-dimensional 13C CP MAS NMR experiments were employed. The peak positions, or chemical shifts, as well as their line widths are indicative of local structure and heterogeneity, respectively. Spectral editing techniques aid in the analysis of features in the regions of severe overlap, particularly for the aliphatic carbons. Relaxation measurements help provide insight into the local dynamics of the system. In this report, we show that the substitution of the hinge region with the polyalanine stretch results in the expected increase in α-helical content. However, changes to the hydrophobic domains were also observed, bringing new light to the question of protein folding in elastin-based polypeptides.

EXPERIMENTAL PROCEDURES

The procedure for preparation of cDNA constructs and for elastin polypeptide expression and purification has been described in detail elsewhere (13, 14). Briefly, DNA constructs were transformed into BL21 cells, and single colonies were inoculated in 2×YT (tryptone 16 g/L, yeast extract 10 g/L, NaCl 5 g/L, pH, 7.0 ± 0.2) medium with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C overnight. This culture was then re-amplified in 2×YT containing 2% glucose and ampicillin for 3.5 h (A600 0.8–1.0) before adding isopropyl-β-
-thiogalactopyranoside (0.1 mM) to induce protein expression. After a 4-h incubation at 37 °C, the bacterial culture was harvested by centrifugation for 10 min at 7500 × g. The cell pellet was digested with cyanogen bromide in 70% formic acid at room temperature overnight followed by dialysis (3.5K cutoff, Pierce) against water for 24–36 h. Elastin polypeptides were purified from this mixture by Sephadex G-25 (Amersham Biosciences) chromatography, eluting with 20 mM sodium acetate, followed by chromatography using a Sepharose SP (Amersham Biosciences) ion-exchange column eluted with 80 mM NaCl in 20 mM sodium acetate. All the polypeptides were desalted on a Sephadex G-25 column and lyophilized before final purification by reversed-phase high-performance liquid chromatography 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 quadrupole time-of-flight mass spectrometry using the facilities of the Advanced Protein Technology Centre, Hospital for Sick Children.

Molecular weights were determined by sedimentation equilibrium at the Division of Molecular and Structural Biology, Ontario Cancer Institute, Toronto, Ontario, Canada. Elastin polypeptides EP20-24-24 and EP20-24-24[23U] were prepared at 25 μm in 50 mM Tris buffer, pH 7, containing 1 mM CaCl2 and 1.5 mM NaCl. The samples were run at 4 °C at 4 speeds (35,000, 40,000, 45,000, and 47,000 rpm) on a Beckman Optima XL-I analytical ultracentrifuge. Global analysis of the data fitted to a single ideal species yielded an apparent molecular mass of 13,754 Da for EP20-24-24 and 11,819 Da for EP20-24-24[23U]. The ratios of the apparent molecular weights measured by sedimentation equilibrium to calculated molecular weights (essentially identical to molecular weights obtained by mass spectrometry) were 0.809 for EP20-24-24 and 0.700 for EP20-24-24[23U]. Residuals showed a random distribution indicating that the fit was good. The data indicate that the samples consisted of monomers under these solution conditions.

Data were acquired on a Varian Unity Inova WB 400 spectrometer, equipped with a 4-mm double-resonance MAS probe (Chemagnetics/Varian NMR, Fort Collins, CO). Typical sam-
ple sizes were ~15 mg for EP20-24-24 and ~20 mg for EP20-24-24[23U]. Data were acquired at 25.0 °C. $^{13}$C chemical shifts were referenced to the tetramethylsilane scale, using hexamethylbenzene as an external standard ($\delta$($^{13}$CH$_3$) = 17.0 ppm at room temperature). Spectra were processed with 20 Hz line-broadening, unless otherwise indicated.

For CP, a 5.0-μs $^1$H 90° pulse was followed by a 1.0- or 1.5-ms contact time with a 5-s recycle delay. Spin locking fields of $\gamma$B$_1$/2$\pi$ = 50 kHz were used for all experiments, including determination of $^{13}$C T$_{1p}$ values and buildup curves. Typical applied field strengths for high power decoupling during acquisition were $\gamma$B$_1$/2$\pi$ = 70–80 kHz. Data were acquired with continuous wave decoupling during acquisition. The spinning speed used in MAS experiments was 8 or 14 kHz, as indicated. $^{13}$C T$_1$ values were obtained by the method of Torchia (26).

To obtain relaxation values for the resolved sites, the peak area (carbonyl) or peak height (all others) were used. The intensities were plotted as a function of time and then fitted to an exponential decay function. Typically, a single-exponential decay was adequate. To plot the buildup curves, the intensities for each resolved peak were first normalized to the tallest height (or greatest area) obtained for that resonance; i.e. the tallest peak for each chemical shift was set to 1.000, and the other slices were normalized accordingly. The buildup curves were then plotted to obtain a qualitative measure of the behavior of the corresponding carbon(s) as a function of CP contact or mixing time.

Pulse sequences for spectral editing are included in work by Zilim and coworkers (27). For the current study, the cross-polarization with depolarization (CPD) sequence was used to identify non-protonated and methyl carbons, suppressing methines (CH) and methylenes (CH$_2$). Optimal spectral editing parameters were determined as 140-μs spin-locking time on $^1$H, 250-μs spin-locking time on the $^{13}$C, cross-polarization time of 1.5 ms, and a depolarization time of 200 μs. These values are similar to those reported previously (28).

**RESULTS AND DISCUSSION**

$^{13}$C Isotropic Chemical Shifts Are Consistent with Previously Reported Data for Native Elastin Samples and Illustrate the Two-domain Makeup of the Polypeptides—Fig. 1 (A and B) shows representative $^{13}$C CPMAS NMR spectra of EP20-24-24 and EP20-24-24[23U]. The backbone carbonyl carbons were identified as a feature with its highest intensity at 172.6 ppm. In EP20-24-24[23U], an additional feature was resolved at 176.6 ppm. The broader resonance centered at ~129 ppm was due to the aromatic carbons of, e.g. the phenylalanines. In the aliphatic region, peaks were resolved at 58, 53, 48, 43, 30, 25, 19, and 16 ppm.

The backbone carbonyl line shapes may be examined in greater detail by using a deconvolution subroutine, as shown in Fig. 2. By inspection, the simplest and best fit deconvolutions were found using three components, i.e. major contributions at 172.2 and 176.5 ppm and a minor contribution at 180 ppm. In EP20-24-24, the relative populations of the 172.2- and 176.5-ppm components were 66 and 27%, respectively. In EP20-24-24[23U], the relative numbers were 52% (for 172.2 ppm) and 39% (176.5 ppm). Small amounts of $\alpha$-helical carbons were also found at 180 ppm for both EP20-24-24[23U] (9%) versus EP20-24-24 (6%). Typical chemical shifts for residues in $\alpha$-helices are found 4–5 ppm downfield of the ones in $\beta$ sheet structures (29–31). Hence, the downfield contributions at 176.5 and 180 ppm were assigned to the $\alpha$-helical carbons of the cross-linking domains. Based on the primary sequence of the polypeptides, 136, or 67.2%, of the 201 amino acids in EP20-24-24 and EP20-24-24[23U] were located in hydrophobic domains, and the remainder, or 32.8%, were found in cross-linking domains.

Therefore, it appears that the populations found for EP20-24-24 are, in this sense, expected for these sequences. Upon substitution of the hinge region, however, the amount of $\alpha$-helicity was greatly increased. This result is somewhat analogous...
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In Addition to the Expected Increase in α-Helical Alanines, an Aliphatic Peak Is Identified in Difference Spectra of 13C CPMAS NMR Data between EP20-24-24 and EP20-24-24[23U]—Although the chemical shifts of all major peaks in EP20-24-24 and EP20-24-24[23U] are largely identical, there are some significant differences in the relative intensities. Fig. 1C is a representative difference spectrum obtained by subtracting EP20-24-24 from EP20-24-24[23U]. The spectra are “normalized” by setting the intensities of the aromatic feature equal, and thus, they cancel out in the difference spectrum. The number of aromatic residues in EP20-24-24 and EP20-24-24[23U] are equal, and it is reasonable to assume that their CP dynamics are also similar, so this approximation appears valid.

The difference spectrum of Fig. 1C includes features in the backbone carbonyl region as well as in the upfield aliphatic region. The EP20-24-24[23U] spectrum has more intensity at the downfield backbone carbonyl population at ~176 ppm. EP20-24-24[23U] has more α-helical content than EP20-24-24 (14), so the appearance of this downfield feature is reasonable. Difference peaks at 53 and 16 ppm also correspond to the α-helical alanines of EP20-24-24[23U].

In addition to the features corresponding to α-helical alanines, a significant difference peak also appeared at 25 ppm. Only Ala was present in greater amounts in the EP20-24-24[23U], and the 176-, 53-, and 16-ppm peaks noted above were present with roughly equivalent intensities. Furthermore, the average chemical shift of Ala methyls is 19.06 ± 2.47 ppm, with only two solved structures with Ala methyls near 25 ppm (36). Hence, it appears most likely that the 25-ppm peak is due neither to Ala nor to any other difference in composition.

This difference peak more likely results from a significant difference in mobility and possibly the structure of a residue type other than alanine. Other carbons that have average chemical shifts near 25 ppm include Gln-Cβ (29.23 ± 2.31 ppm), Ile-Cγ1 (27.72 ± 3.40 ppm), Leu-Cγ (26.81 ± 2.19 ppm), Leu-Cβ1 (24.69 ± 2.58 ppm), Leu-Cβ2 (24.18 ± 2.60 ppm), Lys-Cγ (24.98 ± 2.23 ppm), and Pro-Cγ (27.38 ± 2.99 ppm) (36). The 25-ppm difference peak has roughly the same intensity as the one corresponding to the Ala-Cβ at 16 ppm. As illustrated in Table 1, there are 43 Ala methyl groups in EP20-24-24 and 53 in EP20-24-24[23U]. Even though CPMAS data are not strictly quantitative due to differences in CP efficiencies and other considerations, it is reasonable to use the relative intensities of the two upfield peaks in the difference spectra and tentatively conclude that the number of sites for each is roughly equal; i.e., roughly 10 13C sites contribute to this line shape. If considered on its own, then Gln is too dilute. All of the remaining amino acids in EP20-24-24 and EP20-24-24[23U] (29–32, 35).

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Acid types to be considered, Ile, Leu, Lys, and Pro, contain eight or more carbons (per peptide) in this chemical shift range. Other difference peaks include a minor one centered at ~172.6 ppm and unresolved features downfield of 176 ppm, most likely indicative of possible changes in CP efficiencies and/or structure. In a typical difference spectrum for samples such as EP20-24-24 and EP20-24-24[23U], where there has been substitution, one would expect positive and negative peaks with equal magnitude; i.e., as one component is removed, another is added. However, it appears that the EP20-24-24[23U] carbons have greater CP efficiency over the entire sample, because there is no such “negative” peak in the backbone carbonyl region. Furthermore, small negative peaks are found in the aliphatic region of the difference spectrum. Again, it is purported that the small positive and negative peaks are likely due to differences in CP efficiency between the two samples, because their intensities (both positive and negative) vary with the contact time. In contrast, the difference peaks at 176, 53, 25, and 16 ppm are found in numerous CP experiments and are significantly more intense than the other, minor (difference) peaks. Hence, we focus most effort on the determination of the identities of these features.

$T_1$ Measurements Reflect No Major Differences between Domain Types on the Time Scale of Seconds—$^{13}$C $T_1$ values for the resolved carbons are given in Table 2. The backbone carbonyls and downfield aliphatic carbons of the 43- to 60-ppm region were fit using a single-exponential decay. For the upfield aliphatic carbons, slightly better fits were obtained using double-exponential curves, although a single-exponential decay is a reasonable, though rough, approximation. Presumably, the severe overlap of the various carbon types led to this two-component behavior. Generally speaking, the $T_1$ values for each resolved peak were compared between the two samples, and only very subtle differences were found. For instance, the $T_1$ value for backbone carbonyl at 173 ppm was ~28 s for EP20-24-24 and ~26 s for EP20-24-24[23U]. The C$_\alpha$ carbons at 58 ppm had $T_1$ values of ~20 s. At 53 ppm, largely due to the $\alpha$-helical carbons of alanine, the $T_1$ was 16.5 s in EP20-24-24 and 13.9 s for EP20-24-24[23U]. For side-chain aliphatic carbons at 25 and 30 ppm, the respective $T_1$ values obtained using the single-exponential decay were 4.6 and 6.8 s for EP20-24-24 and 5.8 and 6.2 s for EP20-24-24[23U]. Finally, the methyl carbons at 16 and 19 ppm have $T_1$ values of <1 s. All values are typical for lyophilized samples of elastin and $\alpha$-elastin (20, 34) and elastin mimetics (32). With uncertainties of 1–2 s or less, these values are virtually identical, so there are negligible differences on this time scale.

$^{13}$C $T_{1p}$ Measurements Reflect Increased Mobility of the Hinged Peptides in Both Types of Domains in the Millisecond Regime—Table 3 shows $^{13}$C $T_{1p}$ values for the resolved carbons in the aliphatic region. These relaxation time constants were

### Table 1
Amino acid composition of EP20-24-24 and EP20-24-24[23U]

The number of each residue is shown for each domain type for the two peptides EP20-24-24 and EP20-24-24[23U] (indicated as “[23U]” in the table). Relative amounts are also indicated as “% composition.”

| Amino acid | Hydrophobic region | Cross-linking region, Exon 21/23 | Percent composition | No. of methyl groups |
|------------|---------------------|-----------------------------------|---------------------|---------------------|
|            | Exon 20 | Exon 24 | [23U] | [23U] | Exon 20 | Exon 24 | [23U] | [23U] | EP20-24-24 | EP20-24-24[23U] |
| Ala        | 1       | 1       | 16    | 16    | 26     | 36     | 21.4  | 26.4  | 43     | 53     |
| Gin        | 0       | 0       | 0     | 0     | 4      | 4      | 2     | 2     | 0      | 0      |
| Glu        | 0       | 0       | 0     | 0     | 2      | 2      | 1     | 1     | 0      | 0      |
| Gly        | 15      | 15      | 32    | 32    | 8      | 4      | 27.4  | 25.4  | 0      | 0      |
| Ile        | 2       | 2       | 2     | 2     | 0      | 0      | 2     | 2     | 8      | 8      |
| Leu        | 0       | 0       | 4     | 4     | 8      | 8      | 4     | 4     | 0      | 0      |
| Lys        | 0       | 0       | 0     | 0     | 2      | 2      | 2     | 2     | 0      | 0      |
| Pro        | 5       | 5       | 18    | 18    | 6      | 4      | 14.4  | 13.4  | 0      | 0      |
| Ser        | 1       | 1       | 0     | 0     | 0      | 0      | 0.5   | 0.5   | 0      | 0      |
| Thr        | 0       | 0       | 0     | 0     | 4      | 2      | 1     | 1     | 4      | 2      |
| Tyr        | 0       | 0       | 0     | 0     | 2      | 2      | 1     | 1     | 0      | 0      |
| Val        | 9       | 9       | 28    | 28    | 4      | 2      | 20.4  | 19.4  | 82     | 78     |

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### Table 2
$^{13}$C $T_1$ measurements of EP20-24-24 and EP20-24-24[23U]

$^{13}$C $T_1$ values (in s) of the resolved carbons in EP20-24-24 and EP20-24-24[23U] determined using the CP-with-T1 method of Torchia. Results obtained with a single-exponential decay are shown.

| Chemical shift | EP20-24-24 | EP20-24-24[23U] |
|---------------|------------|----------------|
| ppm           | s          | s              |
| 173           | 28.2 ± 2.0 | 25.9 ± 1.2     |
| 58            | 20.3 ± 4.0 | 20.5 ± 2.5     |
| 53            | 16.5 ± 1.3 | 13.9 ± 0.8     |
| 48            | 12.4 ± 1.4 | 14.6 ± 1.4     |
| 43            | 13.7 ± 1.8 | 16.0 ± 2.0     |
| 30            | 6.8 ± 1.1  | 6.2 ± 0.6      |
| 25            | 4.6 ± 0.8  | 5.8 ± 0.8      |
| 19            | 0.8 ± 0.1  | 0.8 ± 0.1      |
| 16            | 0.8 ± 0.1  | 0.6 ± 0.1      |

### Table 3
$^{13}$C $T_{1p}$ measurements of the aliphatic carbons of EP20-24-24 and EP20-24-24[23U]

$^{13}$C $T_{1p}$ values (in ms) of the resolved carbons in EP20-24-24 and EP20-24-24[23U]. Data were fit using a single-exponential decay function.

| Chemical shift | EP20-24-24 | EP20-24-24[23U] |
|---------------|------------|----------------|
| ppm           | ms         | ms             |
| 58            | 4.0 ± 0.4  | 7.8 ± 1.0      |
| 56            | 4.4 ± 0.6  | 8.2 ± 0.6      |
| 53            | 4.3 ± 0.4  | 8.2 ± 0.6      |
| 48            | 3.0 ± 0.2  | 4.4 ± 0.4      |
| 43            | 2.0 ± 0.1  | 2.8 ± 0.2      |
| 30            | 2.5 ± 0.2  | 3.9 ± 0.3      |
| 25            | 3.4 ± 0.3  | 6.6 ± 0.6      |
| 19            | 12.9 ± 1.1 | 17.3 ± 1.1     |
| 16            | 13.6 ± 1.1 | 20.3 ± 1.2     |
determined to obtain a sense of the differences in dynamics on the millisecond time scale, as opposed to $T_1$ values. Note here that the carbonyl signal did not decay significantly for the selected delay times, indicating that the $\text{^{13}C T}_1$ of the backbone was $\approx 30$ ms. For C$_\alpha$ carbons found in the region of 43–60 ppm, $\text{^{13}C T}_1$ values were 2.0–4.4 ms for EP20-24-24 and 2.8–8.2 ms for EP20-24-24[23U]. For the upfield side-chain aliphatic carbons 25 and 30 ppm, respective $\text{^{13}C T}_1$ values were 3.4 and 2.5 ms for EP20-24-24 and 6.6 and 3.9 ms for EP20-24-24[23U]. Finally, for the resolved methyl carbons at 16 and 19 ppm, $\text{^{13}C T}_1$ values were 13.6 and 12.9 ms for EP20-24-24 and 20.3 and 17.3 ms for EP20-24-24[23U]. Generally, the carbons of EP20-24-24[23U] had longer $\text{^{13}C T}_1$ values than EP20-24-24. In some cases, such as the 43-ppm peak, the difference between the two samples was $<1$ ms. The increase in $\text{^{13}C T}_1$ values over the entire spectrum was also consistent with the higher CP efficiencies in EP20-24-24[23U] suggested earlier. The longer $\text{^{13}C T}_1$ values in EP20-24-24[23U] again suggest that this polypeptide is much more rigid than EP20-24-24. More significant, however, is the conclusion that the rigidification of the structure was not localized to the cross-linking domains, where the substitution of the hinge region had taken place. Instead, the changes in millisecond dynamics occurred across the sample, including the hydrophobic domains.

$\text{CP Buildup Curves Provide Additional Insight into the Identity of the 25-ppm Difference Peak}$—As CP efficiency may be considered another measure of relative motion, CP buildup curves were constructed for each of the resolved peaks in EP20-24-24 and EP20-24-24[23U], as shown in Figs. 3 and 4. Each buildup curve shows measured peak intensity as a function of CP contact time and is typical for solid proteins. Fastest buildup in signal intensity in CP experiments was observed for methylene and methine carbons, because the C–H couplings were strongest in these sites. As expected, the C$_\alpha$-carbons and other non-methyl side-chain carbons had the highest intensities at short CP times. In contrast, the nonprotonated carbons underwent CP via the dipolar couplings to its nearest neighbor or next-nearest neighbors. In proteins, the backbone carbonyls would have significantly smaller intensities than the upfield aliphatics at short contact times. Methyl carbons have a high number of directly bound C–H pairs. However, the motion of the methyl rotor severely mitigates the dipolar coupling, so these buildup curves tend to be intermediari between the nonprotonated and the CH/CH$_2$. Generally, the “front-end” of the buildup curves, or the relative intensities at short CP times, tend to have slightly higher intensities for the EP20-24-24, compared with EP20-24-24[23U]. At longer times, the intensities of the EP20-24-24 peaks decrease, which is expected, given the shorter $\text{^{13}C T}_1$ values reported in the previous section. In essence, these buildup curves are consistent with the $\text{^{13}C T}_1$ measurements.

Interestingly, however, these buildup curves provide additional insight into the identity of the 25-ppm peak that appeared in the difference spectrum of Fig. 1C. Fig. 5 shows the buildup curves obtained for EP20-24-24 and EP20-24-24[23U] for the peaks at 30, 25, 19, and 16 ppm. The peaks at 19 and 16 had chemical shifts that are typical for methyl carbons, as noted earlier. The methyl peaks for EP20-24-24 had nearly identical buildup curves. A similar result of overlapping buildup curves was also obtained for the methyls of EP20-24-24[23U]. The buildup curves of 25 and 30 ppm may be interpreted somewhat
analogously. Most of the intensity of the 30-ppm peak was due to Cβ-Val, a methine carbon. The 25-ppm peak may be due to a methylene or methine peak of one of the hydrophobic amino acids (35, 36), as noted above. In addition, some methyl groups are found in this region (35, 36). Although the 25- and 30-ppm peaks in EP20-24-24 have very similar buildup curves, they appear to be different in EP20-24-24[23U], namely, that the 25-ppm peak has buildup curve behavior that appears intermediary between the “mostly methine” and methyl peaks. One possible interpretation is that the 25-ppm peak of EP20-24-24[23U] has more methyl character than that of EP20-24-24.

**Spectral Editing Results Provide Additional Support for the Role of Methyl Groups in the Rigidification of All Domains in EP20-24-24[23U]**—Spectral editing based on spin temperature and CP dynamics was demonstrated first by Zilm and coworkers (37, 38) and was shown more recently to be a good tool for the analysis of peptides and proteins (28). Briefly, a “full” spectrum showing all peaks is obtained with CPMAS. A “subspectrum” of only methylene peaks is obtained with cross-polarization with polarization inversion. Nonprotonated and methyl carbons are identified using cross-polarization with depolarization (CPD). In CPD subspectra, the methine and methylene peaks are suppressed.

Fig. 6 shows the CPD subspectra and deconvolution of the methyl region for these two samples. A four-component fit was used for this analysis. The majority of the EP20-24-24 methyls were found at 19.4 ppm (65%), with minor contributions at 15.8 ppm (28%), 24.6 ppm (5%), and 11.6 ppm (2%). In contrast, fewer of the EP20-24-24[23U] methyls were observed at 19 ppm (40%). The populations at 15.6 ppm (37%) and 24.2 ppm (17%) were noted, with a small population at 11.2 ppm (6%). The ratios of upfield (16 and 11–12 ppm) to downfield (24–25 and 19 ppm) peaks are roughly consistent with the values determined for deconvolution of the backbone carbonyl line shapes.

Removal of the hinge region and replacement with alanines causes putative changes in packing of methyl groups, as reflected in the changes in overall line shape. Based on the deconvolution of these edited spectra, EP20-24-24[23U] contains more α-helical alanines, a conclusion that is supported by the analysis of the backbone carbonyl line shape and the difference spectra. In addition, methyl groups with resonance frequencies in the 19-ppm range in EP20-24-24 presumably adopt different conformations in EP20-24-24[23U], resulting in the increased intensity of the 24- to 25-ppm feature.

The question arose as to whether the 25-ppm feature is a fraction of the population that does not CP well in the EP20-24-24 (but does in the unhinged), or if there is a change in local environment or conformation that is reflected by a shift in peak position. Pursuing this ambiguity, the EP20-24-24 was also observed at ~30 °C. No change in the methyl line shape was observed at this lower temperature. Therefore, it is most likely that the increase in
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25-ppm peak intensity in the EP20-24-24 is due to a conformational change of a population of methyl groups.

The line width of the major component at 19 ppm decreases from 550 Hz in EP20-24-24 to 400 Hz in EP20-24-24[23U]. Although still relatively broad, this difference is, again, consistent with the picture of greater disorder in EP20-24-24. Overall, there is considerable disorder in the two polypeptides, with natural line widths of all components in these line shapes of ~3–4 ppm, with the possible exception of the 19-ppm peak in EP20-24-24, as noted above.

The assignment of the ~25-ppm peak is further clarified with these editing results. As shown in Fig. 1, the peak at 25 ppm in the full CPMAS spectrum of EP20-24-24[23U] has significantly higher intensity than EP20-24-24. However, an unambiguous assignment is complicated by the fact that it is a peak in a crowded region of the $^{13}$C spectrum, and the sample is unenriched. Initial support for the assignment of the 25- to 26-ppm peak to the methyls of leucine and possibly isoleucine is found in the literature (35, 36) and in our separate measurements of Leu and Pro-Ile peptides (not shown). To clarify, we attempted to distinguish the identity of this peak from its CP dynamics, because it appears to be more like a methyl than the other side-chain aliphatics, as shown with the buildup curves. Finally, the line shape information provided in the CPD subspectra lend even more support to the conclusion that the 25-ppm peak is likely due to methyls from the hydrophobic domains. Here we note that no differences were observed between EP20-24-24 and EP20-24-24[23U], using cross-polarization with polarization inversion (not shown), again lending more support to the notion that the 25-ppm difference peak is due to a change in the methyl populations. Although any one of these results (editing, chemical shifts, or buildup curves) may not make for a compelling argument on its own, together they appear to provide enough evidence for the assignment of the 25-ppm peak to the leucine methyls. Finally, we note that the nature of this assignment does not completely eliminate the possibility that the 25-ppm peak is due to another site, such as the Cβ-Ala or one of the methylene peaks mentioned earlier. However, our data indicate that the most probable candidate is the aforementioned Leu methyl carbon(s).

Concluding Remarks—The classic approach to discussions of the structure and functional models of elastin has focused much effort into understanding the organization of the hydrophobic domains, particularly in the repeating polypeptides. However, these experiments show that the two domain types are not independent. Rather, changes in the central turn or hinge of the cross-linking domains affect the structure and, particularly, the dynamics of the hydrophobic regions.

The substitution in the hinge region results in the expected increases of peak intensities corresponding to α-helical Ala. However, we find that there is also a significant difference in intensity at 25 ppm. To identify this peak, the chemical shift databases and peptide compositions were used in this analysis. In addition, CP buildup curves were recorded, and spectral editing experiments were conducted. At this point, the most likely assignment of this peak is the methyl carbons of isoleucines or leucines, which are found only in the hydrophobic domains. There is a small possibility that the 25-ppm peak may be attributed to another carbon, such as the methyls of the alanines. The only truly definitive way to answer this question would be isotopic enrichment of selected amino acids, such as the Ala. However, based on the collection of evidence, the assignment of the 25-ppm peak to the methyls found only in hydrophobic domains seems reasonable and likely.

In addition to the 25-ppm peak and its implications, replacement of the hinge region was concurrent with a change in $^{13}$C T$_{1p}$ values, although T$_{1}$ values reflected few differences. The relaxation times are consistent with the observations of differences in CP properties between the samples. Overall, these NMR results indicate that the EP20-24-24 polypeptide undergoes slightly more rapid motion than the unhinged peptide.

These experiments indicate that EP20-24-24 has greater flexibility and a slightly larger degree of heterogeneity than the unhinged EP20-24-24[23U]. It is extremely likely that these two characteristics are related; i.e., the greater flexibility of the hinged polypeptide EP20-24-24 allows a larger number of conformations to be accessed. As noted earlier, this type of conformational distribution is a recurring motif in elastin peptides and in the native protein and is perhaps one of the key features of biological elasticity. This flexibility seems particularly relevant when considering the phenomenon of coacervation or aggregation in this system. Possibly, when this hinge is replaced by a polyalanine stretch, the number of interactions between hydrophobic domains of a given peptide chain are greatly reduced. Instead, these hydrophobic domains interact with those of a neighboring strand or chain, and, as a result, aggregation or coacervation is facilitated. This model is compatible with observations of coacervation temperature; i.e., EP20-24-24[23U] coacervates at lower temperatures than EP20-24-24 (14).

Although significant effort has been placed in determining the structural characteristics of the hydrophobic repeats of elastin, this study provides yet more evidence that the sequences in cross-linking domains also play a key role in determining the overall structure of the protein. Indeed, these results lay the groundwork for a compelling new set of experiments that test the interactions between domain types and suggest that a refined model for elastin structure function is long overdue.

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