Elastin Recoil is Driven by the Hydrophobic Effect

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Abstract

Elastin is an extracellular matrix material formed when the precursor protein, tropoelastin, undergoes coacervation and crosslinking. It is found in all vertebrates where its reversible elasticity, robustness and low stiffness are essential for the normal function of arteries, lungs, and skin. It is among the most resilient elastic materials known: During a human lifetime, arterial elastin undergoes in excess of 10^9 stretching/contracting cycles without being replaced or repaired and its slow oxidative hardening has been identified as one of the 'hard limits' on human lifespan. The physical basis for these properties, most notably the molecular mechanism of entropic recoil, have been a source of controversy for over fifty years. Herein, we report a combined NMR and thermomechanical study of elastin that establishes the hydrophobic effect as the primary driver of elastin function. Water ordering at the solvent:protein interface was directly observed as a function of stretch at the molecular level using double quantum filtered ^{2}H NMR and the most extensive thermodynamic analysis of elastin function performed to date was obtained by measuring elastin length and volume as a function of applied force and temperature in normal water, heavy water and with added co-solvents. When elastin is stretched, the heat capacity increases, water is ordered proportional to the degree of stretching, elastin's internal energy decreases, and heat is released in excess of the work performed. Together, these properties show that spontaneous recoil in elastin at physiological temperatures and conditions is primarily driven by the hydrophobic effect. Consistent with this conclusion are decreases in the thermodynamic signatures when co-solvents that alter the hydrophobic effect are introduced. Hydrophobic effect-driven recoil, as opposed to a configurational entropy mechanism where hardening from crystallization can occur, may be the origin of elastin's unusual resilience.

Significance

Elastin is found in vertebrate tissues such as arteries, lungs, and skin that require elasticity. Elastin's key properties are high reversibility, low stiffness and great robustness. It is among the most resilient elastic materials known and elastin mimetics are promising targets for regenerative medicine. However, the molecular mechanism of entropic recoil is not well understood. Elastin is formed from a very hydrophobic protein with an equivalent masse of water and the controversy is whether the driving force for recoil derives from the entropy gain of the

protein and/or the water. We used NMR to demonstrate that water in the elastin matrix is progressively ordered upon stretching and used a mechanical device to demonstrate that the thermodynamic properties of elastin recoil are those of the hydrophobic effect, differing from the known properties of rubber. We conclude that elastin recoil is primarily driven by the hydrophobic effect and we suggest that this accounts for elastin's low stiffness and high resilience.

Introduction

Although elastin has been identified as one of the 'hard limits' on human lifespan (1), its molecular mechanism of entropic recoil in is not well-understood and has been a source of controversy for over fifty years (2-12). A widely accepted hypothesis is that elastin is like rubber and an increase in the polymer's configurational entropy drives recoil (2, 3, 13). Unlike rubber, elastin gains elasticity only when swollen with an approximately equal mass of water (14) and it's physiological function requires different mechanical properties: First, elastin recoil is fully reversible on the timescale of a heartbeat. This enables the elastic energy stored in arteries when the heart contracts to be fully recovered when the heart relaxes between beats. Second, elastin is highly compliant, with a Young's modulus (~ 0.6 MPa) that is an order of magnitude less than a typical rubber (~ 10 MPa) (15). Third, elastin is far more robust than rubber (16), undergoing more than 10^9 expansion/contraction cycles without appreciably lengthening of during a normal human lifespan. and failure of any of these properties results in hypertension which in turn causes vascular calcification, ventricular hypertrophy, renal dysfunction and stroke.

The elastic matrix is formed when tropoelastin, one of the most hydrophobic proteins found in nature, is exported to the extracellular matrix and undergoes a liquid-liquid phase separation followed by enzymatic cross-linking (17, 18). Tropoelastin is organized in alternating proline-rich hydrophobic domains and alanine-rich crosslinking domains. (Figure 1a) (19). In 1970, Weis-Fogh (20) found that the amount of heat released when elastin is stretched is in large excess over the applied work (ΔG) and concluded that, unlike rubber, (21, 22) elastin's internal energy decreases when stretched (4, 9, 23). It was hypothesized that the solvent exposed surface area of hydrophobic droplets increases when deformed and ordering of water at the hydrophobic surface of elastin droplets is increased, i.e, spontaneous recoil is driven by the hydrophobic effect. Flory and Hoeve rejected the droplet model (2) in favor of a rubber-like network and

pointed out that the thermodynamic analysis of Weis-Fogh (4) had neglected the contribution to the change in internal energy from a volume change that could occur when the sample is stretched, and they found that elastin's volume and internal energy are constant when stretched in aqueous glycol (3:7) or 30% PEG. They concluded that elastin is like rubber, i.e. configurational entropy of the polymer drives recoil (2, 3). However, the their analysis neglected the effect of glycol or PEG on water:protein interactions (24, 25) and it was subsequently reported (9) that elastin's volume is constant when stretched in water.

In this report, stretch-induced ordering of water (^2H_2O) in the elastin matrix was quantified using double quantum (2Q) filtered ^{2}H NMR, (26, 27) and a complete thermodynamic study of elastin's mechanical properties was obtained. The NMR experiment uses the property that isotropic molecular tumbling in bulk water (^2H_2O) averages the ^{2}H quadrupole coupling to zero whereas tumbling of water at a hydrophobic surface is anisotropic and the coupling is non-zero. Using the methods of multiple quantum spectroscopy, the bulk water signal is eliminated from detection and only the 2Q signal from ordered water is observed. (28) We have determined elastin's thermodynamic properties with a custom-made thermomechanical apparatus. The length and volume of a purified elastin sample were measured with a high-resolution camera as a function of applied force and temperature (from 3°C to 55°C) in several aqueous solvents from which virial equations of state were constructed and the relevant thermodynamic properties, ΔG, ΔS, ΔH, ΔU and ΔCp, were calculated as functions of temperature and strain. Of particular interest are the dependencies of the heat capacity, enthalpy and entropy with temperature and strain. For example, signature features of the hydrophobic effect when a hydrophobic surface is brought into contact with water are an increase in the heat capacity (29) and large, compensating changes in both ΔH and $T\Delta S$ resulting in a small net driving force, ΔG. (30)

Results

Reversibility and the equation of state.

Figure 1a depicts the domain structure of bovine elastin (747 residues) and Figure 1b depicts a scanning electron micrograph demonstrating that the fibrous structure of natural elastin has been preserved in the purification procedure. (31) A requirement in our thermodynamic analysis is reversibility of the length following changes in the applied force or temperature.

Figures 1c and 1d show that elastin's length subjected to stretch/contract and heat/cool cycling is fully reversible on the time scales of ~5 minutes for length changes and 3-5 minutes for temperature changes. From 3°C to 55°C, the decrease in length is non-linear and reaches a temperature-independent length above ~50 °C, Figure 1d, and the variation of *l* with *F* is linear as previously seen. (9) The *l(F,T)* surface, Figure 1e, was obtained by combining multiple *l(F)* and *l(T)* data sets and fitting 308 data points (elastin in water) to equation 6. The RMSD of 0.26 mm is comparable to the estimated accuracy of the length measurements. Fit parameters for elastin in water and in other solvents are listed in S1.

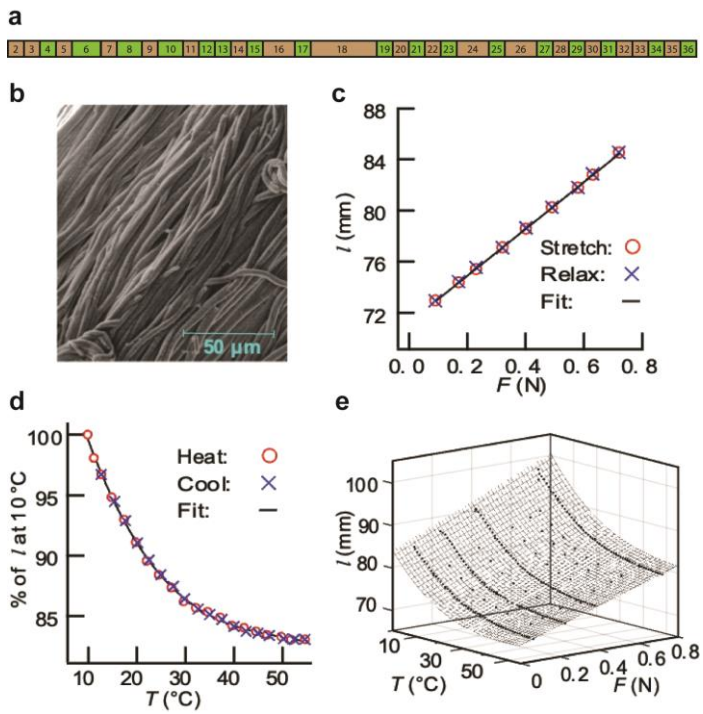

Figure 1. (**a**) Domain architecture of bovine elastin. Brown rectangles represent hydrophobic domains and green rectangles represent crosslinking domains. (**b**) SEM of purified bovine elastin showing the uniform fibrous structure found in natural elastin. (**c**) Reversibility of the fiber length from two stretch and contract cycles. The fit to two cycles (line) is linear. (**d**) Reversibility from heating and cooling. The fit (line) is a polynomial 3rd-order in *T*. (**e**) Surface plot, *l(F,T)*, obtained by combining data from multiple *l* vs. *F* and *l* vs. *T* experiments. The fit (lines) is a polynomial 1st-order in *F* and 3rd-order in *T*.

Elastin's mechanical and thermodynamic properties in water and comparison to natural rubber.

The thermo-mechanical parameters obtained from purified elastin in water at 10°C, 35°C and 45°C are summarized in Figure 2. The Young's modulus E, which depends on l_{rlx} and k_H, has a minimum value of 0.38 MPa at 20°C in water (Figure 2a). The range of values determined here, 0.38 MPa to 0.42 MPa, using a large sample with many fibers is in good agreement with the value of 0.45 MPa previously determined from small (5 micrometer) single-fiber samples (23). The temperature dependence of E results from a force constant that increases linearly as temperature is increased and the relaxed length that decreases with temperature in a non-linear way reaching a constant value at temperatures above ~50°C (Figure 2b).

Elastin and rubber tension (force per unit area, f) measured at constant T and P can be directly compared. Tension in elastin is less than that of rubber and varies linearly with strain (Figure 2c). With equation 3, f can be separated into entropic and enthalpic components ($f = f_S + f_H$) (13, 23). Values of f and f_S were calculated for elastin using equations 7-9 with the virial coefficients in S1 and from the data of Shen, *et.al.* (21) for rubber. The ratios of f_S/f for elastin at 30°C and 50°C are depicted as a functions of strain and compared to rubber at 30°C (Figure 2d) (21). The horizontal line, $f_S/f = 1$, represents and ideal elastomer in which case the force is entirely entropic. At 30°C and strains greater than 20%, rubber is approximately ideal ($f_S/f \sim 0.8$) whereas elastin is far from ideal at all strains ($f_S/f > 4$). When the temperature of elastin is raised to 50°C, the entropic contribution to the total tension approaches that observed in rubber. Because the entropic tension is large compared to the net tension at physiological temperatures, the entropic and enthalpic tensions are opposed in elastin and the magnitude of the enthalpic tension, $f - f_S$, is almost as large as the entropic component and decreases in parallel with f_S as the temperature is increased.

Elastin's thermodynamic properties as functions of strain and temperature are depicted in Figures 2e and 2f. The expressions used to obtain ΔG, ΔH, ΔS and ΔCp from the virial equation of state, are derived in the materials and methods section and apply to an open system at constant temperature and pressure that is in equilibrium with the surrounding water.

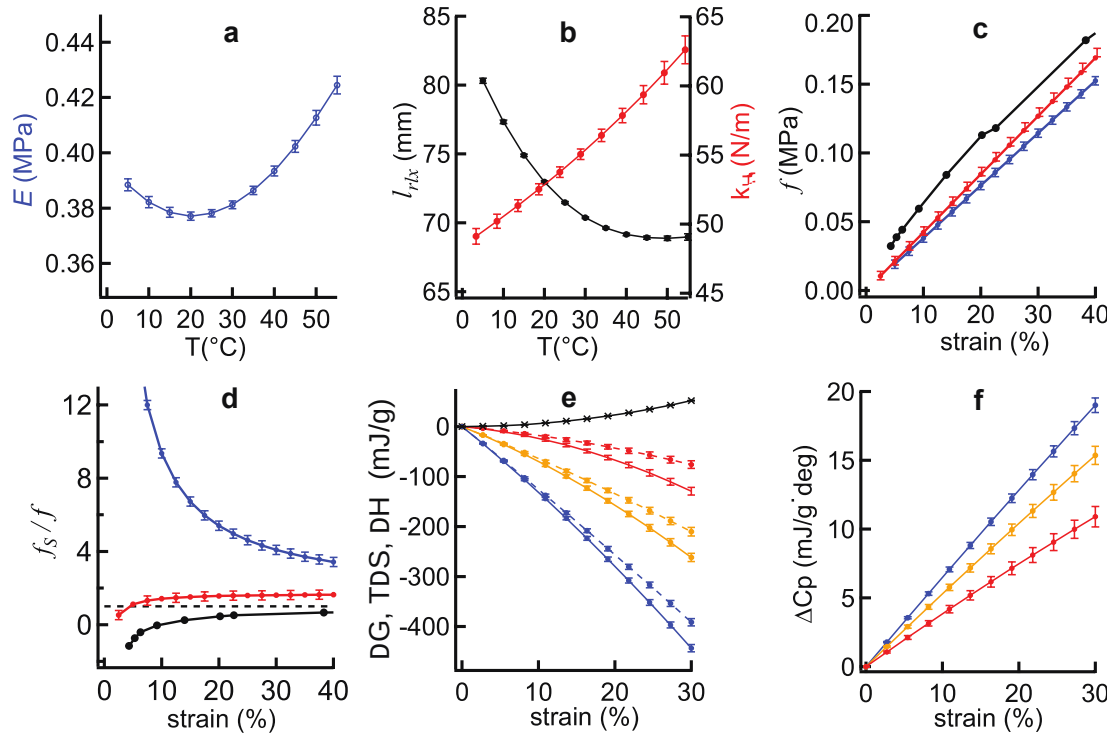

Figure 2: Temperature dependence of elastin's mechanical and thermodynamic properties. (**a**) Young's modulus, (**b**) the relaxed length, l_{rlx} (■), and the force constant, k_H (■). (**c**) Tension, *f*, as a function of strain for elastin at 30°C (■), elastin at 50°C (■) and rubber at 30° (■). (**d**) Ratio of the entropic tension to the total tension, f_S/f, as a function of strain for elastin at 30°C (■), elastin at 50°C (■) and rubber at 30°C (■). (**e**) Elastin *ΔG* (■), *ΔH* (⋯) and *TΔS* (−) vs strain at 10°C (■), 25°C (■) and 45°C (■). *ΔG* values at 10°C, 25°C and 45°C overlap**.** (**f**) Elastin ΔC_p vs strain at 10°C (■), 25°C (■) and 45°C (■).

The thermodynamic parameters determined in this way are for the entire system which, as shown below, changes in hydration as the temperature and sample length are varied. Importantly, the heat to work ratios *ΔH/ΔG* determined at 23°C using our method when elastin is stretched to strains of 10%, 20% and 30% are -23±0.3, -11.9±0.3, and -8.1±0.2, respectively, which are in agreement with values previously determined calorimetrically (9).

Elastin's thermodynamic properties are fully consistent with the conclusion that stretch increases the exposure of elastin's predominantly hydrophobic residues to water and this contributes to the net thermodynamic force that drives recoil: when elastin is stretched from the relaxed state, *ΔH* and *TΔS* are negative at all values of strain and temperature and their small net

difference, ΔG, gives elastin it's physiologically important property of high compliance (low Young's modulus). The magnitudes of ΔH and $T\Delta S$ decrease substantially as the temperature is raised while ΔG is nearly independent of temperature. At temperatures below 45°C, spontaneous recoil in elastin is endothermic and entropy driven with a free energy change that is less than the heat liberated. Elastin's heat capacity (Figure 2f) has not been previously determined and we find that ΔCp increases when strain is applied. The pattern of changes in ΔG, ΔH and $T\Delta S$ (Figure 2e) and the increase in heat capacity (Figure 2d) are all characteristic features of the transfer of hydrophobic substances into an aqueous environment (29, 30, 32). The decrease in the amount of bound ordered water, and its concomitant decrease in volume, are expected consequences of the hydrophobic effect.

Volume expansion and water uptake is small when elastin is stretched.

Because heat is in large excess of the work performed when stretched, it was concluded that, unlike rubber, elastin's internal energy decreases when stretched (4, 9). McCrum *et al.*, in a series of papers, pointed out that this conclusion neglected a change in volume that could occur when elastin is stretched and proposed that the release of heat from water uptake associated with an increased volume could account for the large release of heat when elastin is stretched (2, 5-7). However, they subsequently reported that the increase in elastin's volume when extended by 20% was negligible (9).

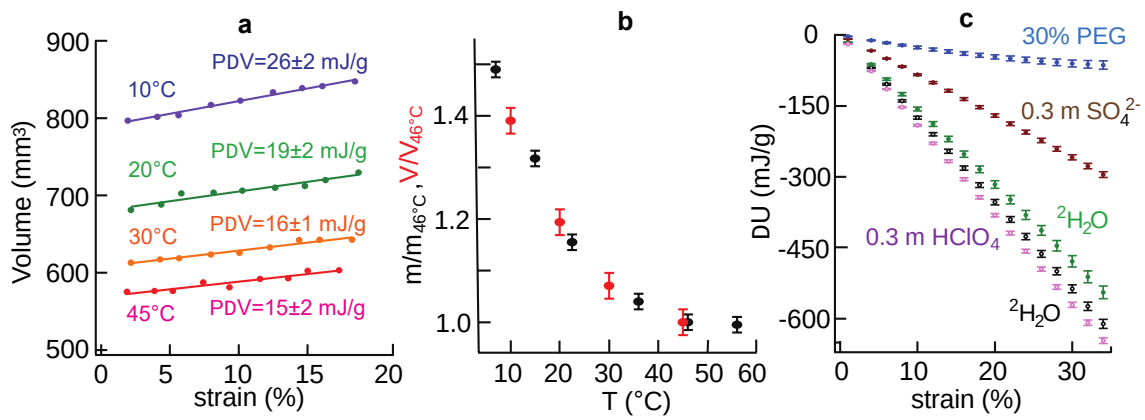

Figure 3: (**a**) Elastin volume as a function of strain and temperature. Lines are linear fits to the volume vs strain data and the indicated values of $P\Delta V$ are per gram of protein dry weight at 15% strain. (**b**) Temperature dependence of the mass (•) and volume (•) of elastin samples normalized to their values at 46°C. (**c**) Strain induced values of ΔU for elastin at 20°C in the indicated solvents.

To resolve this disagreement, we measured elastin's volume as a function of strain and temperature, Figure 3a, and applied the thermodynamic relation $\Delta U = \Delta H - P\Delta V$. Volume was estimated from the digital images of the sample The coefficients of expansion calculated from the volume data, 14.8×10^{-3} at 15°C and 3.4×10^{-3} at 38°C, are in good agreement with previous determinations (10). At temperatures from 10°C to 45°C, elastin's volume expands by 5% to 6% when stretched isothermally to a strain of 15%. At 37°C, the change in internal energy from expansion (-15±2 mJ/g) is small compared to that from enthalpy (-144.5±6.3 mJ/g) and the reduction in internal energy (-159.5± mJ/g) is, in fact, large compared to the stretch work (35.5±0.3 mJ/g). As temperature is increased, heat loss is reduced and at 50°C, the magnitudes of the enthalpy change, and work are comparable (Table 1). Like the effect of temperature, the internal energy loss is also reduced when co-solvents, 0.3 m sodium sulfate or 30% PEG, are added (Figure 3c).

Elastin's volume increases by a large amount, 40%, when cooled (Figure 3a) and we find that the mass and volume of an elastin sample vary in direct proportion with one another (Figure 3b). We conclude that the increase in volume when elastin is stretched or cooled is due entirely to the influx of water, i.e., the changes in the equilibrium volume, dV_{eq}, and the number of moles

of water of hydration, dn_{H_2O}, are related by $dV_{eq} = w_{H_2O} dn_{H_2O}$ where w_{H_2O} is the partial molar volume of water. With the known dry mass of the vacuum-dried sample, elastin is 48% and 63% water by weight at 45°C and 10°C, respectively.

Co-solvents and solvent deuteration alter elastin's mechanical and thermodynamic properties.

To further confirm the conclusion that the hydrophobic effect is the primary driver of elastic recoil we have studied the effects of cosolvents on elastin. Sodium sulfate and 20 kDa PEG primarily affect the water:protein interface and don't bind to proteins while 0.3 molal sodium perchlorate binds weakly at the backbone and has little effect on water properties. Sodium ion, a weak Hoffmeister ion, is expected to be less significant.(33, 34) Sulfate increases the surface tension of water which increases the energy required to create the cavity at a hydrophobic surface (35-37) and 20kDa PEG is excluded from the protein surface which increases the protein's thermodynamic activity (38-40), both of which destabilize protein conformations with exposed hydrophobic surface area and promote hydrophobic burial (35, 41). A more subtle change in water's properties is caused by water deuteration (42)(43, 44). Compared to ^1H_2O, the surface tension is slightly lower and the solubility of nonpolar solutes in heavy water is higher in ^2H_2O (45-47). The temperature dependence in each solvent of the mechanical parameters and the strain dependence of the thermodynamic parameters are depicted in Figure 4 and S2-S4.

With the addition of Na_2SO_4 or PEG, which affect elastin at the solvent:protein interface, the characteristic features of elastin in water are significantly altered due to a 7% reduction in the relaxed length and a 12% increase in the force constant. The entropic force that opposes stretch and the attendant heat released are both decreased, the increase in heat capacity is diminished, however, the net driving force is nearly unchanged. For example, when elastin is stretched by 25% at 25°C in 30% PEG, the heat released decreases from 375 mJ/g to 10 mJ/g, the increase in ΔC_p is reduced from 17 mJ/K to 2 mJ/K and the decrease in internal energy is comparable in magnitude to the stretch work. In qualitative terms, elastin's thermodynamic properties in sulfate or PEG are similar to those of elastin in water at temperatures above 45°C.
Compared to sulfate or PEG, elastin is substantially less affected by 0.3 m $NaClO_4$ which binds weakly to the protein backbone and does not increase water's surface tension. Moreover, the

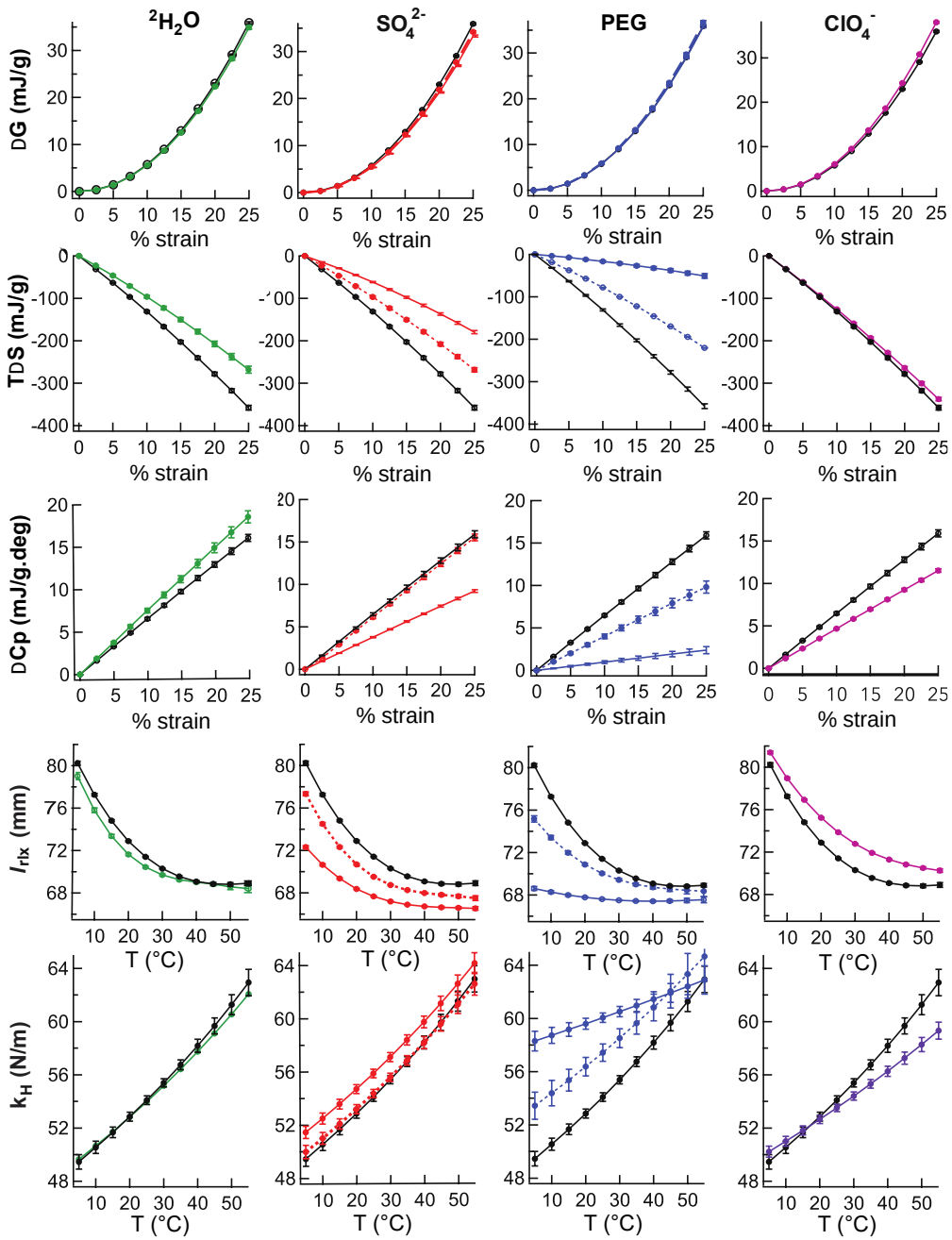

Figure 4: Thermomechanical properties of elastin in different aqueous solutions. Columns in the figure are the different solvents: ^2H_2O (–), Na_2SO (··· 0.1 m and – 0.3 m), 20 kDa PEG (··· 15% and – 30%) and $NaClO_4$ (–0.3 m). Rows in the figure are thermodynamic parameters (*DG*, *TDS* and *DCp*) are plotted versus % strain at 25°C and the mechanical parameters (l_{rlx} and k_H) are plotted versus temperature. For reference, the data from elastin in pure water (–) is shown in each graph.

mechanical parameters change in the opposite directions. This is expected since adding negative charge to an otherwise neutral protein increases repulsion resulting in an increased relaxed length and a decreased force constant. The small changes in the mechanical and thermodynamic properties are fully consistent with studies which show that cross-linked elastin is highly disordered and lacks significant 2° structure (48-51) – weak perchlorate binding to the backbone cannot significantly disrupt a disordered protein.

When stretched in ^2H_2O, k_H and l_{rlx} are smaller than in ^1H_2O, $T\Delta S$ is less negative, ΔC_p rises faster and ΔG is unchanged. Similarly, when folded proteins are denatured in ^2H_2O rather than ^1H_2O, an increase in ΔC_p with constant ΔG (entropy-enthalpy compensation) is usually observed and this has been associated with an increase in solvent exposed, non-polar surface area (42, 52). These results are fully consistent with the greater solubility of non-polar molecules in ^2H_2O (53) and show that small changes in the strength of the hydrophobic effect have observable effects on the thermodynamics of elastin stretch and recoil. These surface effects are more important than, for example, stronger H-bonds in the protein which should have the opposite effect on the stretch ΔH. This is consistent with our NMR studies indicating a near absence of stable 2° structure in elastin (48, 54) and molecular dynamics simulations of an aggregated elastin-like peptide (11) indicating that putative β–turns stabilized by H-bonds have sub-nanosecond lifetimes.

Each of these cosolvents alters the thermodynamics of stretching in elastin in a manner consistent with recoil primarily driven by the hydrophobic effect.

Water is ordered when elastin is stretched.

The thermodynamic experiments described above provide strong thermodynamic evidence for the primacy of the hydrophobic effect in elastin recoil. We next set out to directly observe water ordering – the hallmark of hydrophobic effect - upon stretching of elastin (30). To determine if water ordering in elastin varies with strain, we used double-quantum (2Q) filtered ^{2}H NMR of elastin in ^2H_2O. In this experiment, the signal from bulk water which reorients isotropically is removed and the 2Q signal from ordered water which reorients anisotropiocally and has a non-zero quadrupole coupling is detected. The experiment is quantitated in terms of the water fraction, f_o, that is ordered. Note that elastin's mechanical properties in ^2H_2O are almost the same as in ^1H_2O, Figure 4. We find that the ordered water fraction, 2% in relaxed elastin,

increases substantially to 81% when the sample is stretched by 27% (Figure 5a). This large increase is direct observation of the hydrophobic effect at work during elastin function.

Next, we observed the effects of cosolvent on water ordering: in 10% PEG, the ordered fraction is reduced ~2-fold compared to pure ^2H_2O at each stretch (Figure 5b) and further lowered in 0.1m sodium sulfate (Figure 5c). In 0.5m sodium sulfate, ordered water is almost eliminated from observation in this experiment (Figure 5c). Overall, these results show that the amount of ordered water decreases with the addition of cosolvents that reduce solvent exposed surface area: Na_2SO_4 and PEG.

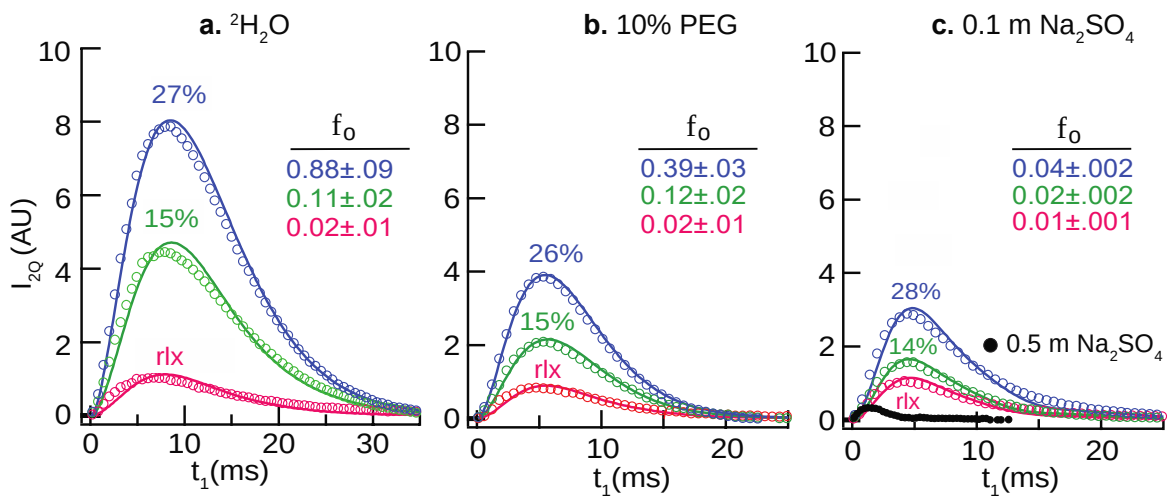

Figure 5: Plots of the deuterium 2Q NMR signal intensity, I_{2Q}, as a function of the 2Q preparation time, t_1. Open circles are the intensities of the 2Q filtered NMR signal with only every third data point shown. The ordered fraction, f_o, was determined by least squares fitting (solid lines) to equation 13. Elastin fibers were hydrated in (**a**) ^2H_2O, (**b**) 10% PEG (w/w), (**c**) 0.1 m Na_2SO_4 and 0.5 m Na_2SO_4 at 28% stretch (black dots). The fibers are relaxed (o), 15-20% stretched (o), and 30-35% stretched (o).

Discussion

To understand the molecular basis of recoil in elastin, we have combined a complete study of elastin's thermodynamics and measured water ordering in the elastin matrix at the molecular level. Under physiological conditions, elastin is a non-ideal elastomer. At 37°C, the entropic force that drives recoil, $-T\Delta S$, is five-fold greater than the magnitude of the net driving force, ΔG. The entropic and the net driving force, ΔG, are approximately equal in rubber, as is the case for an ideal elastomer (22). Because $-T\Delta S$ and the enthalpy change, ΔH, are opposed (opposite sign), elastin has high compliance. Otherwise, a heart with greater contractive force would be required to expand arteries. From Chatelier's principle, the large heat absorption with recoil follows directly from the large decrease in the relaxed length when the temperature is raised. At 50°C, the relaxed length reaches a constant value and both $T\Delta S$ and ΔH are drastically reduced from their values at 5°C, 820±xx mJ/g and 790±xx mJ/g to 56.3±19 mJ/g and 18.1±19 mJ/g, respectively. Since the variation in ΔG, over the same range of temperature is small, −26±2 mJ/g, there is significant enthalpy-entropy compensation. This and the stretch induced increase in the heat capacity, also observed here, are signature features of the hydrophobic effect.

With thermo-mechanics, we have confirmed that the heat released from stretch is in large excess over the total work performed, previously determined using a calorimeter (4, 9). In addition, we have determined that this process is fully reversible, Figure 1c. Thus, objections to this finding based on a time-scale argument (5) can be disregarded. Because ΔH from stretch is large and negative, Weis-Fogh concluded that, unlike rubber (22), elastin's internal energy had decreased. Flory, however, observed no change in the volume or internal energy when elastin was stretched in 30% PEG and concluded that elastin is like rubber (2). While Weis-Fogh had assumed that volume was constant when stretched, Flory had assumed that PEG has negligible effect on water:protein interactions. To resolve this, we have measured elastin's volume as a function of temperature and strain and used the general relation, $\Delta U = \Delta H - P\Delta V$, to determine ΔU in pure water and in 30% PEG. We concur that ΔV in 30% PEG is negligible, however, ΔV is small in water and the contribution to the internal energy is less than 10% of ΔH, Figures 2 and 3. Thus, in physiological conditions, ΔU decreases when elastin is stretched. The difference between elastin in water and 30% PEG is primarily the difference in ΔH and not ΔV.

Table 1: Thermodynamic properties of elastin recoil from in water at three temperatures. Sample strain was 25%.

property	25°C	37°C	50°C
$\Delta \boldsymbol{G}, \mathbf{mJ/g}$	-36.3±.3	-35.5±0.3	-38.0±.51
$-\boldsymbol{T}\Delta\boldsymbol{S}, \mathbf{mJ/g}$	-360.4±7.2	-180.4±7.3	-56.3±19.
$\Delta \boldsymbol{H}, \mathbf{mJ/g}$	324.2±7.2	144.5±6.3	18.1±19
$\Delta \boldsymbol{C_p}, \mathbf{mJ/g \cdot K}$	-16.0±0.4	-12.2±0.7	-7.04±1.7
$\Delta \boldsymbol{U}, \mathbf{mJ/g}$	342±12.3	159.5±11.8	32.1±21.5
$\boldsymbol{P}\Delta\boldsymbol{V}, \mathbf{mJ/g}$	-18±2	-15±2	-14±2

To better understand the effects of co-solvents, we have determined elastin's thermo-mechanical properties and the ordered water fraction, f_o, with three co-solvents: sodium sulfate and PEG change the water:protein interface and promote protein compaction and don't bind to the protein while the perchlorate binds to the protein leading to protein expansion and has little effect on water properties. These expectations are fully borne out in changes to elastin's thermo-mechanical properties, Figure 4, and to changes in the ordered water fraction, f_0, Figure 5. The relaxed length decreases by a large amount in sulfate or PEG and increases by a small in perchlorate. ΔG is essentially unchanged by any of the co-solvents while the magnitudes of $T\Delta S$, ΔH and ΔC_p are all significantly and progressively decreased by large amounts in sulfate and PEG but almost unchanged in perchlorate. In 30% PEG, ΔH and ΔC_p approach zero, $\Delta G \sim -T\Delta S$, (S3) and the ordered water fraction in the NMR experiment is greatly reduced.

Conclusions

At physiological temperature, recoil in elastin is characterized by several unusual properties: (i) the internal energy increases, (ii) the heat capacity decreases, (iii) the entropic driving force is large compared to the net driving force because spontaneous recoil is highly endothermic and (iv) stretching elastin orders water. Properties (ii), (iii) and (iv) are characteristic of a spontaneous process driven by the hydrophobic effect (30, 32) and all of these

properties are different or absent in rubber where the driving force for recoil is the increase in the polymer's configurational entropy (21, 55). We conclude that at physiological temperatures and in the absence of co-solvents, the predominant driving force for recoil in elastin is the hydrophobic effect.

Natural and artificial configurational entropy springs such as rubber suffer from strain crystallization while experiencing repeated strain cycles, undergoing microscopic phase transitions that result in zones of ordered material with low compliance.(56) Hydrophobic effect-driven recoil may be the origin of elastin's unusual resilience in that it offers a pathway to avoid these phase transitions by retaining disorder even while extended, enabling elastin to be more self-healing than rubber.

Materials and Methods

Thermodynamic properties from the temperature dependence of Elastin's mechanical properties.

Elastin immersed in water is an open system in equilibrium with the surrounding solvent at constant temperature and pressure and the relevant thermodynamic expression is the Gibbs free energy (30)

$$dG = -SdT + VdP + Fdl + \mu dn. \quad \text{equation 1}$$

In equations 1, F is force, l is the sample length and, respectively, n and μ are the number of moles and chemical potential of water in the elastin matrix and other variables have their usual meaning. It is shown, Figure 3c and the discussion therein, that when the elastin matrix is at osmotic equilibrium with the surrounding water (eq), the changes in elastin's volume and moles of water are related by $dn_{H_2O} = w_{H_2O}^{-1} dV_{eq}$, where $V_{eq}(l,T)$ is the equilibrium volume. It is shown in S5 that after incorporating this relation into equation 1, the following Maxwell relation is obtained,

$$(\partial S/\partial l)_{T,P,eq} = -(\partial F/\partial T)_{l,P,eq}. \quad \text{equation 2}$$

The entropy change is obtained by integrating both sides of equation 2,

$$\Delta S_{T,P,eq} = -\int (\partial F(l,T)/\partial T)_{l,P,eq}\, dl. \quad \text{equation 3}$$

and $\Delta G_{T,P,eq}$, the non-$P\Delta V$ work, is obtained by integrating the stretch force, F,

$$\Delta G_{T,P,eq} = \int F(l,T) dl, \text{ equation 4}$$

With equations 3 and 4, the enthalpy and heat capacity, $\Delta H_{T,P,eq}$ and ΔC_p, are readily obtained using the relations, $\Delta H = \Delta G + T\Delta S$, and $\Delta C_p = (\partial \Delta H_{T,P,eq}/\partial T)_P$. Flory obtained an equation (13) that separates the total stress (f) into enthalpic (f_H) and entropic (f_S) components which are. respectively, the first and second terms on the RHS of equation 6,

$$f = (\partial H/\partial l)_{T,P,eq} + T(\partial f/\partial T)_{l,P,eq}. \quad \text{equation 5}$$

To experimentally determine $F(l,T)$, the sample length, l, was measured from 3°C to 55°C with forces from 0 N to 0.72 N at each temperature. The length was found to accurately fit a virial equation of state that is 1st-order (linear) in $\tilde{F} \equiv F - \bar{F}$ and 3rd-order in $\tilde{T} \equiv T - \bar{T}$.

$$l(\tilde{F},\tilde{T}) = \mathrm{b}_{00} + \mathrm{b}_{10}\tilde{F} + \mathrm{b}_{01}\tilde{T} + \mathrm{b}_{11}\tilde{F}\tilde{T} + \mathrm{b}_{02}\tilde{T}^2 + \mathrm{b}_{03}\tilde{T}^3. \quad \text{equation 6}$$

Equation 6 is a Taylor series expansion about the averages of the experimental force $\bar{F}$ and $\bar{T}$. The coefficient b_{12} was zero within experimental error and not included in equation 6. With the definition of the length at zero force,

$$l_{rlx} \equiv \mathrm{b}_{00} + \mathrm{b}_{01}\tilde{T} + \mathrm{b}_{02}\tilde{T}^2 + \mathrm{b}_{03}\tilde{T}^3 - \left(\mathrm{b}_{10} + \mathrm{b}_{11}\tilde{T}\right)\bar{F}, \quad \text{equation 7}$$

and the spring constant,

$$k_{\mathrm{H}} = (\mathrm{b}_{10} + \mathrm{b}_{11}\tilde{T})^{-1}, \quad \text{equation 8}$$

equation 6 can be solved for F and written in the form of Hooke's law,

$$F = k_H(l - l_{rlx}). \quad \text{equation 9}$$

Note that both k_{H} and l_{rlx} are temperature-dependent and are related to the Young's modulus by the relation $E = k_H l_{rlx}/a$. These mechanical properties provide greater physical insight than the Young's modulus alone and all thermodynamic properties can be calculated from them. Equations for the thermodynamic properties are summarized below and detailed derivations are given in the supplemental data. The free energy change is,

$$\Delta G_{T,P,eq} = \frac{1}{2}k_{\mathrm{H}}\Delta l^2, \quad \text{equation 10}$$

the entropy change is,

$$\Delta S_{T,P,eq} = -\frac{1}{2}\left(\frac{\partial \mathrm{k_H}}{\partial T}\right)_l \Delta l^2 + k_H\left(\frac{\partial l_{rlx}}{\partial T}\right)_l \Delta l, \quad \text{equation 11}$$

and the change in heat capacity is,

$$\Delta C_p = -\frac{1}{2}\hat{T}\left(\frac{\partial^2 k_H}{\partial T^2}\right)_l \Delta l^2 + \left[k_H\hat{T}\left(\frac{\partial^2 l_{rlx}}{\partial T^2}\right)_l + k_H\left(\frac{\partial l_{rlx}}{\partial T}\right)_l + \hat{T}\left(\frac{\partial l_{rlx}}{\partial T}\right)_l\left(\frac{\partial k_H}{\partial T}\right)_l\right]\Delta l. \quad \text{equation 12}$$

Elastin sample preparation

A 0.24 g dried elastin fiber (~ 77 mm × 1.6 mm × 6.4 mm) was cut from purified, bovine neck ligament. The purification procedure,(31) avoids harsh conditions used in older procedures, removes microfibrils, collagen and preserves fibrillar structure of native elastin, Figure 1a. A single sample was used for all of the thermomechanical data reported in the table SI and Figures 6a, 6c, 7 and 9. The sample was glued (Elmer's E616 Super Glue) to 1/4" diameter rods at both ends for attachment to the stretcher apparatus. A separate and larger sample was used to determine the temperature dependence of elastin's mass, Figure 6b. With the larger sample (dry mass of 2.029g), the contribution to the total mass from water on the surface of the sample was minimized. After removal from the temperature-controlled water bath, samples were quickly blotted on a chem-wipe before weighing. Additional water loss that occurs on sample warming was minimized by performing the experiments in the cold room.

Stretcher Instrument.

The sample compartment of the apparatus, S6, used to determine $F(l,\mathrm{T})$ and $V(l,\mathrm{T})$ was machined from delrin (sides and base) and plexiglass windows (front and back) through which the fiber was photographed with a 36-megapixel digital camera (Sony A7r). The short rod glued to the lower end of the elastin sample was fixed to the base and the top rod was attached to a string that was passed over a low friction pulley (Super pulley, ME9450a, Pasco, Roseville, CA) to an adjustable mass that was converted to force using the acceleration due to gravity, 9.8 m/s^2. The camera and tank were rigidly fixed to a table, from which the fiber length was measured as a function of applied force and temperature with an accuracy of 0.1mm to 0.2mm using ImageJ software (https://imagej.nih.gov/ij/). The digital image was calibrated against a 200 mm ruler placed next to the sample. The temperature controller (not shown) regulated to within 0.1°C of the set point with a PID controller and the temperature dependence was measured with a platinum resistance thermometer placed in the sample compartment. To avoid thermal gradients, solvent was continuously circulated from the top to the bottom of the tank by a miniature pump.

Volume as a function of strain and temperature.

The sample volume was determined directly from the digital image. Combined with the length measurement, the sample width along the length of the elastin sample was also measured and the sample thickness, the smallest dimension, was calculated from the width measurement with the assumption that the thickness to the width ratio was the same as in the dry sample.

Thermo-mechanical data collection and analysis.

Length versus force and temperature data were collected as separate *l(F)* runs at 9 forces from 0.09N to 0.72N and *l*(*T*) data was collected at 1°C intervals from 2°C to 55°C. The data were combined into a single table with a typical size of 300 *l*(*F,T*) values from which thermo-mechanical properties were calculated using the two Matlab scripts listed in the Supplemental data. With the first script, the *F(l,T)* data were least-squares fit to equation 5 to determine the virial coefficients, b_{ij}, from which all thermo-mechanical properties and their standard errors were calculated using the second script. Standard errors were determined using a Monte Carlo procedure that propagates the effects of random errors in the experimental measurements of the sample length to the thermo-mechanical properties at each temperature and strain. Initially. the random errors in *l* were assumed to be normally distributed (57) with the standard deviation equal to the RMSD of the polynomial fit, typically 0.2 mm to 0.3 mm, which is essentially the same as our estimate of the experimental accuracy of the instrument. Simulated data sets were fit in the same way as the experimental data set from which distributions of the thermo-mechanical properties at each temperature and strain are determined and the standard deviations of these distributions are the confidence intervals and shown as the error bars in graphs of the thermo-mechanical properties. Monte Carlo calculations were converged with 1000 simulated data sets. Alternatively, the virial coefficients, b_{ij}, rather than the sample lengths, *l*, were randomized using the standard deviations from the least-squares fit and error bars determined in this way were typically two-fold larger. To avoid over interpreting the thermo-mechanical data, these were used in Table 1, SI Table 1, Figures 2-4 and Figures 6-8.

Strain induced changes in solvent ordering studied by double quantum ^2H NMR.

We have observed the effect of stretch on ordering of ^2H_2O in the elastin matrix. Insofar as molecular reorientation of ^2H_2O is anisotropic, as is the case for ordered water, ^2H_2O has a non-zero ^2H quadrupole coupling and the "forbidden" double quantum (2Q) NMR transition can be observed using the methods of indirect detection. (28) Because reorientation in bulk water is

isotropic, the quadrupole coupling is zero, the 2Q transition is absent and it's NMR signal can be removed by 2Q filtration and only the 2Q transition is observed. (26, 27) To determine the effect of strain on the fraction of ordered water in the sample, f_o, we observed the buildup of the 2Q NMR signal from ordered water, I_{2q}, as a function of the 2Q preparation time, t_1. The NMR signal is proportional to f_o and is well-described by the relation, (26)

$$\frac{I_{2q}(t_1)}{I_{1Q}^{tot}} = \mathrm{f_o} e^{-2R_2 t_1} \int_0^{\pi} sin^2 \left[\frac{\omega_Q t_1}{2}(3cos^2\theta - 1)\right] sin\theta d\theta. \text{ eq. 13}$$

The ordered fraction, f_o, and the residual quadrupole coupling, $\nu_Q = \omega_Q/2\pi$, were determined by a two parameter least-squares fit of $I_{2q}(\mathrm{t_1})$ to equation 13 with I_{1Q}^{tot}, the 1Q signal from all of the ^2H_2O in the sample, and R_2, the transverse relaxation time, constrained to the values separately determined in Hahn echo experiments with and without 2Q filtration, S7 (26).

2Q filtered ^{2}H spectra were collected on a homebuilt, 11.6 T instrument with a solenoidal coil probe (58) (90° pulse width = 4.2 μs) and the previously described pulse sequence and phase cycle (26). ^{2}H spectra (2k complex points) were obtained with a 5 kHz offset and 128 equally spaced 2Q preparation times (t_1). Spectra were Fourier transformed in the direct dimension and peak heights of the 2Q filtered signals are displayed, Figure 10, as a function of the 2Q preparation time, t_1. Suppression of the 1Q signal to a level below the noise was confirmed by collecting a 2D spectrum using the same phase cycle, however, the preparation time was fixed (5 ms – 10 ms) and the indirect dimension was the time between preparation and conversion of the 2Q coherence during which both the 1Q and 2Q signals evolve (26). With a 5 kHz offset, peak heights in the indirect dimension at the 2Q frequency (10 kHz) and the 1Q frequency (5 kHz) were, respectively, large and below the noise level.

NMR sample preparation.

Both ends of a purified elastin fiber (~20 mg and ~1mm × ~12 mm) were superglued to ~12 mm lengths of 1/16" diameter plastic rod, equilibrated overnight in the desired aqueous solution and inserted into a 3 mm NMR tube (length ~30 mm) after removal of excess solvent by blotting on paper. With the elastin sample centered in the NMR tube, one rod was sealed and held stationary to the tube with glue and the other rod was sealed and held in place with parafilm

which could be removed so that the fiber could be stretched to a different length and resealed. Fiber lengths were measured with a digital caliper.

Materials.

Elastin samples were purified from fresh bovine nuchae membranes using a method that preserves the natural fibrous structure and removes other proteins (Figure 1a) (31). Sample solutions were prepared from deionized water with reagent grade solutes (Na_2SO_4, 20 kDa PEG, and $NaClO_4$). The experiments in deuterated solvent used 99% ^2H_2O (Cambridge Isotopes, Andover, MA).

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Abbreviations: NMR, nuclear magnetic resonance; 2Q, double quantum; PEG, polyethylene glycol

Data availability statement: All data used herein (Figures 1, 3a, 3b, 5 and tables 1 and SI table of all data is available at https://academicworks.cuny.edu/cc_pubs/985/. All of the thermomechanical data has been distilled into the virial coefficients listed in SI Table 1 from which properties in Figures 2, 3c and 4 were calculated as described in the materials and methods sections.

Code availability statement: The custom Matlab scripts used to calculate the virial coefficients from the raw thermodynamic data and the thermomechanical properties and their associated confidence intervals from the virial coefficients are listed in the supplemental information.

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