Tropoelastin is the soluble precursor of elastin that bestows tissue elasticity in vertebrates. Tropoelastin is soluble at 20 °C in phosphate-buffered saline, pH 7.4, but at 37 °C equilibrium is established between soluble protein and insoluble coacervate. Sedimentation equilibrium studies performed before (20 °C) and after (37 °C) coacervation showed that the soluble component was strictly monomeric. Sedimentation velocity experiments revealed that at both temperatures soluble tropoelastin exists as two independently sedimenting monomeric species present in approximately equal concentrations. Species 1 had a frictional ratio at both temperatures of ~2.2, suggesting a very highly expanded or asymmetric protein. Species 2 displayed a frictional ratio at 20 °C of 1.4 that increased to 1.7 at 37 °C, indicating a compact and symmetrical conformation that expanded or became asymmetric at the higher temperature. The slow interconversion of the two monomeric species contrasts with the rapid and reversible process of coacervation suggesting both efficiently incorporate into the coacervate. A hydrated protein of equivalent molecular weight modeled as a sphere and a flexible chain was predicted to have a frictional ratio of 1.2 and 1.6, respectively. Tropoelastin appeared as a single species when studied by pulsed field-gradient spin-echo NMR, but computer modeling showed that the method was insensitive to the presence of two species of equal concentration having similar diffusion coefficients. Scintillation proximity assays using radiolabeled tropoelastin and sedimentation analysis showed that the coacervation at 37 °C was a highly cooperative monomer-n-mer self-association. A critical concentration of 3.4 g/liter was obtained when the coacervate was modeled as a helical polymer formed from the monomers via oligomeric intermediates.

Elastin forms a highly insoluble cross-linked extracellular matrix that is predominantly responsible for the elasticity of vertebrate tissue. The precursor of elastin, tropoelastin, is devoid of cross-links. Following secretion from the cell surface, tropoelastin undergoes coacervation, which is a process of self-association characterized by an inverse temperature transition (1). Tropoelastin is soluble in aqueous solution at room temperature in vitro, but upon raising the temperature to 37 °C the solution becomes turbid as tropoelastin molecules associate to form large aggregates (2, 3). This process of coacervation results from multiple intermolecular interactions of the hydrophobic domains (3, 4). The tropoelastin coacervate is a thick viscoelastic phase that is not miscible with the overlying solution (5). On cooling to 20 °C, the aggregates dissociate reversibly, and the solution turns clear. Alternating between hydrophobic domains in the protein are short sequences of amino acid residues that form the cross-linking domains (6). Coacervation may concentrate and align tropoelastin molecules prior to elastin formation via lysyl oxidase-mediated cross-linkage of the lysine residues that leads to a growing elastic fiber (4, 7, 8).

Vrhovski et al. (1) demonstrated that maximal coacervation of recombinant human tropoelastin occurs under the physiologically relevant conditions of 37 °C, 150 mM NaCl, and pH 7–8. Through coacervation, tropoelastin molecules were thought to be converted from molecules largely lacking secondary and tertiary structure to a more ordered state (2, 7, 9, 10). Electron micrographs show parallel arrays of 5-nm-wide filaments of tropoelastin coacervates that are similar to the fibrous structure of mature elastin (3, 4, 11). The importance of coacervation in the formation of lysine cross-links has been shown in smooth muscle cells where culturing at temperatures below 37 °C hampers elastin formation (12, 13).

In the work described here, analytical ultracentrifugation, pulsed field-gradient spin-echo (PGSE), nuclear magnetic resonance spectroscopy, and scintillation proximity assays were employed, in conjunction with computer modeling studies, to characterize the thermodynamic and hydrodynamic properties of recombinant tropoelastin before (20 °C) and after (37 °C) coacervation. We show that at both temperatures soluble tropoelastin exists as at least two monomeric forms that have the capacity to coacervate in a manner similar to a classical critical concentration model of polymerization.

MATERIALS AND METHODS
Preparation of Tropoelastin SHEL26A—Tropoelastin SHEL26A (M₆ = 60,140) was expressed and extracted from a culture of Escherichia coli BL21(DE3) as described previously (1, 14). SHEL26A is a recombinant human tropoelastin lacking the hydrophilic domain encoded by exon 26A and is identical to a naturally occurring isoform of tropoelastin. The abbreviations used are: PGSE, pulsed field-gradient spin-echo; PBS, phosphate-buffered saline; T, tesla.
human tropoelastin found in connective tissue. Sodium dodecyl sulfate-polycrylamide gel electrophoresis was performed to check the purity of protein samples (15). Concentrations of acrylamide for the resolving gel and the stacking gel were 10 and 4%, respectively. For coacervation, full-length tropoelastin was dissolved in phosphate-buffered saline (PBS) containing 10 mM phosphate, pH 7.4 and 150 mM NaCl, which mimics the ionic conditions found to be optimal for coacervation (1).

Sedimentation Equilibrium—Sedimentation equilibrium of tropoelastin was performed in a Beckman XL-A analytical ultracentrifuge at different initial loading concentrations ranging from 0.3 to 4.0 g/ml. The protein solution was dialyzed extensively against PBS at 4 °C and spun in a benchtop centrifuge at room temperature at 30,000 rpm for 30 min prior to the ultracentrifuge experiments. 120-mm spinning cups were used. PBS was calculated to be 1.0057 and 1.0008 g/ml at 20 and 37 °C, respectively, for a protomer of unhydrated spherical particle and is defined in Equation 6 and as 1.0057 and 1.0008 g/ml at 20 and 37 °C, respectively (Sednterp version 1.01), and density values as given above. Plots of $s^2_{20,w}$ and $D_{20,w}$ versus initial loading concentration were extrapolated to zero concentration to give the $s^2_{0,w}$ and $D_{0,w}$ values. The molar weight, $M$, of each species was then calculated from the relationship (23) shown in Equation 4,

$$M = s^2_{0,w}RT/\rho D_{0,w}(1 - \eta_{2,0,w})$$  

(Eq. 4)

where $\rho_{2,0,w}$ is the density of water at 20 °C.

Sedimentation data were used to calculate the fractional ratio $f_{2,0,w}/f_{0}$ of tropoelastin, $f_{2,0,w}$ is defined in Equation 5 (23),

$$f_{2,0,w} = M(1 - \eta_{2,0,w})N\rho_{2,0,w}$$  

(Eq. 5)

where $N$ is Avagadro’s number; $f_{c}$ is the frictional coefficient of a hard, unhydrated spherical particle and is defined in Equation 6 and as follows (23),

$$f_{c} = 6\pi\eta R_{c}$$  

(Eq. 6)

$$R_{c} = (3M(4\pi N)^{1/3})$$  

(Eq. 7)

where $\eta$ is the viscosity of water at 20 °C and $R_{c}$ is the radius of the sphere of the particle with molar weight of SHEL262A at 20 °C.

By using values of $f_{2,0,w}$, calculated from Equation 5, $R_{c}$ for sedimenting species of tropoelastin was calculated using an equation analogous to Equation 6. The value of $f_{2,0,w}$ was used to obtain an axial ratio, $a/d$, for a prolate spheroid of revolution (where $a$ represents the major axis and $d$ represents the minor axis) (23).

Nuclear Magnetic Resonance Spectroscopy—Tropoelastin was dissolved in both PBS and pure water, and the solutions were brought to concentrations ranging from 0.5 to 10 g/liter. Samples were loaded in 5-mm outer diameter NMR tubes (Shigemi) to a height of 1 cm. PGSE experiments were conducted on a Bruker DRX-400 spectrometer (Karlsruhe, Germany) with an Oxford Instruments 9.4 T vertical wide bore magnet (Oxford, UK), using a Bruker 10 T/m z axis gradient probe. PGSE experiments conducted at 20 °C used a pulse sequence as described previously (24). Experiments conducted at 37 °C used a dual double spin-echo pulse sequence to compensate for convection in the sample (25). The pulse sequence parameters for all experiments are as follows: duration of field-gradient pulses, $\Delta = 2$ ms; time interval between field-gradient pulses, $\Delta = 20$ ms; $90^\circ$ $R_{p}$ pulse, 18–20 μs; total spin-echo time, TE = 40 ms; between 128 transients per spectrum for the highest concentration sample (10 g/liter) and 4096 transients per spectrum for the lowest concentration sample (0.5 g/liter) were acquired; maximum field-gradient, $g_{max} = 3.75$ T/m. The water signal was suppressed during the relaxation delay to allow the use of higher receiver gain. Routinely, 16 spectra were acquired with a sequential increase in the value of $g$.

The gradients were calibrated using the known diffusion coefficient of water in an isotropic and unbounded region (26). A single experiment was conducted for each concentration of sample at 20 °C. At 37 °C duplicate experiments were conducted on a 10 g/liter sample of tropoelastin in water only. Tropoelastin forms a coacervate in PBS at 37 °C yielding a sedimenting phase in the sample tube (1), thus precluding this type of sample for diffusion measurements at 37 °C.

In each spectrum the signal intensity was measured as the integral versus s

$$s^2 = \text{ln}(r_f)(dr_f/\bar{t})$$  

(Eq. 3)

where $r_f$ is the radial distance of the solution meniscus, and $\bar{t}$ is the equivalent time of sedimentation. The data were best fit by a model describing two independently sedimenting species, and the program returned values of the sedimentation and diffusion coefficients ($s$ and $D$, respectively) for each species along with their standard errors (22).

Values of $s$ and $D$ were corrected to the equivalent values in water at 20 °C ($s_{2,0,w}$ and $D_{2,0,w}$, respectively) using values for the viscosity, $\eta$, of PBS was 1.012 and 0.7035 cm2/s at 20 and 37 °C, respectively (Sednterp version 1.01), and density values as given above. Plots of $s_{2,0,w}$ and $D_{2,0,w}$ versus initial loading concentration were extrapolated to zero concentration to give the $s_{0,w}$ and $D_{0,w}$ values.

$\text{Properties of Human Tropoelastin}$

$\text{Vuong}$

$\text{Data analysis was performed by directly fitting plots of $s(r)/(r/t)$ versus $s^2$ using the DCDT package.}$

$\text{The apparent sedimentation coefficient defined in Equation 3 (21),}$

$$s^2 = \text{ln}(r_f)(dr_f/\bar{t})$$  

(Eq. 3)

$\text{where}$

$\text{is the radial distance of the solution meniscus, and}$

$\text{is the equivalent time of sedimentation.}$

$\text{The data were best fit by a model describing two independently sedimenting species, and}$

$\text{the program returned values of the sedimentation and diffusion coefficients}$

$\text{and}$

$\text{respectively) for each species along with their standard errors (22).}$

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$\text{concentration to give the and values.}$

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$\text{of each species was then calculated from the}$

$\text{relationship (23) shown in Equation 4,}$

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$\text{where}$

$\text{is the density of water at 20 °C.}$

$\text{Sedimentation data were used to calculate the fractional ratio}$

$\text{of tropoelastin,}$

$\text{is defined in Equation 5 (23),}$

$$f_{2,0,w} = M(1 - \eta_{2,0,w})N\rho_{2,0,w}$$  

(Eq. 5)

$\text{where}$

$\rho_{2,0,w}$

$\text{is the density of water at 20 °C.}$

$\text{The Ommenu package of programs for analyzing data using the function or weight average molecular weights is available at}$

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fit a straight line to the plotted data, and the slope of this line provided an estimate of the diffusion coefficient. Diffusion coefficients for tropoelastin in water and in PBS at 20 °C were then plotted as a function of concentration, and linear regression was used again to extrapolate the line to zero concentration thus yielding values for $D_0^b$ and $D_2^b$ for tropoelastin in PBS. The data were then corrected for the viscosity of water and $D^w_{20}$. A weight average $D_{w,av}$ was calculated from the duplicate diffusion coefficients estimated for tropoelastin in water at 37 °C; the weighting factor was the inverse of the variance of each value.

Computer Modeling—Computer modeling was used to address two questions arising from the ultracentrifuge data. First, is the resolution of the PGSE NMR diffusion data adequate to suggest the existence of a more than one species? Second, does a system in which one species is modeled as a compact sphere and the other species as a flexible chain or rigid rod molecule account for the observed hydrodynamic properties?

To answer the first question, a computer model (model I) was written in Maple (Waterloo Software Inc., Canada). Signal intensities for a PGSE NMR diffusion experiment were calculated for a series of gradient values using the same parameters as those used in the actual experiment. The model assumed two independent non-interacting species of equal concentration having $D^w_{20}$ of 4.0 and $6.0 \times 10^{-11}$ m^2/s (comparable to those determined by sedimentation velocity experiments at 20 °C, see “Results”). The following formula, shown in Equation 8, was used for the calculation,

$$E = C_1e^{-D_1^b} + C_2e^{-D_2^b}$$

(Eq. 8)

where $E$ is signal intensity; $C_1$ and $C_2$ are the concentrations of the two species; $D_1$ and $D_2$ are the diffusion coefficients of the two species; and $b$ is the Stejskal-Tanner parameter (27).

The signal-to-noise ratio and its standard deviation were calculated from the actual PGSE NMR experiment. Random noise, whose amplitude had a standard deviation equal to that of the experimental data, was then superimposed on the calculated signal intensities. The natural logarithm of the calculated signal intensity with its associated noise was then plotted as a function of the Stejskal-Tanner parameter $b$. Linear regression was used to fit a straight line to the simulated PGSE NMR data in order to determine whether there was any statistically discernible deviation from linearity.

To answer the second question, a model (Model II) was written in Matlab (MathWorks) and was used to predict $D^w_{20}$ for a tropoelastin monomer, when it was modeled as follows: 1) a hard unhydrated sphere; 2) a hard hydrated sphere; 3) a hydrated flexible chain; and 4) a rigid rod. Note that the aim of the simulation was not to calculate accurate values for $D^w_{20}$ for possible conformations of the protein but rather to determine whether the difference in the experimental values could be explained in terms of these conformations. For this reason, the model assumed an ideal solution in which the diffusing molecules do not interact or give rise to excluded volume effects. The amino acid residues, which constitute the monomers in the flexible chain and rigid rod models, were assumed to be identical hard spherical units and to have the same partial specific volume (0.7574 ml/g) was determined for the protein from its amino acid sequence (see “Materials and Methods”).

The Stokes equation and the Einstein equation (also referred to as the Einstein-Smoluchowski equation (see Ref. 28)) were used to calculate values of $f^{w}_{0,w}$ and $D^w_{20}$, respectively, of the amino acid residues in the flexible chain and rigid rod models, and of the unhydrated and hydrated spheres. The hydrodynamic parameters for the flexible chain were calculated using Equations 36 and 37 of Kirkwood (30) and for the rigid rod using Equations 22 and 23 of Riseman and Kirkwood (30). A weighted average specific volume for the hydrated spheres was calculated to account for the associated water molecules. For a typical protein, each gram of its dry weight contains between 0.3 and 0.6 g of associated water, whose properties are significantly different to those of the bulk water (31). This corresponds to limits of $-1.4$ and $-2.8$ water molecules per amino acid residue for tropoelastin monomers, and thus hydrodynamic parameters were calculated for all models on the basis of these two values. The values of $f^{w}_{0,w}$, $D^w_{20}$ for the hydrated sphere, flexible chain, and rigid rod were calculated as the ratio of the molecule’s $f^{w}_{0,w}$ to that of the hard unhydrated sphere.

Sedimentation Equilibrium—Fig. 1 shows representative results from sedimentation equilibrium experiments performed at 15,000 rpm on tropoelastin in PBS before (20 °C) and after (37 °C) coacervation. Any insoluble coacervate was rapidly sedimented to the base of the solution column and remained undetected. The overlap of the data from the three separate loading concentrations (○, □, and ○) shows that the protein is homogeneous.

Plates (Canberra Packard Co.). $^{125}$I-Labeled tropoelastin was dissolved in PBS at 10 g/liter, and 100 µl of serially diluted solution was placed into duplicate wells. The plate was loaded into a Packard TopCount microplate scintillation and luminescence counter (Canberra Packard Co.) and allowed to equilibrate for 1 h at 20 and 35 °C prior to counting. 35 °C is the highest functional temperature. Association of $^{125}$I-labeled tropoelastin by coacervation brought the radioactive signals in close proximity to excite the scintillator, whereas signals from the uncoacervated material remaining in solution very poorly excite scintillator. γ-Counts were determined for 1 min per well in the low energy range and normal efficiency mode. Cross-talk reduction was applied to reduce signals from neighboring wells. For each set of assays, the duplicate data were averaged. The data at 20 °C were subtracted from those at 35 °C to correct for passive coating of the wells by the protein. The corrected counts at 10 g/liter were normalized to 100%. A plot of the normalized counts versus the total concentration of tropoelastin was then produced from the three independent sets of data.

RESULTS

Sedimentation Equilibrium—Fig. 1 shows representative results from sedimentation equilibrium experiments performed at 15,000 rpm on tropoelastin in PBS before (20 °C) and after (37 °C) coacervation. Any insoluble coacervate was rapidly sedimented to the base of the solution column and was not detected. At both temperatures, the plots of apparent weight average molar weight, $M^w_{av}$, versus concentration, $c(r)$, from three different loading concentrations of protein indicated that the soluble protein was monomeric (e.g. Fig. 1A). Plots of the $\Omega$ function versus $c(r)$ (e.g. Fig. 1B) from the different loading concentrations overlapped closely, indicating that both chemical and sedimentation equilibrium had been attained. Thus, the diffusion was homogeneous, and there were no significant concentrations of incompetent species such as irreversibly formed aggregates or truncated species (16, 17).

Plots of $c(r)$ versus $r^2$ were fit with a model describing a single nonideal species using nonlinear least squares techniques (e.g. Fig. 2A). Values were returned for both the molar weight, $M^w_{av}$, of the species and its nonideality as measured by
the second virial coefficient, \(B\) (Table I). This model adequately fitted the data obtained at both 20 and 37 °C as judged by the random distribution of residuals (e.g. Fig. 2B). Returned estimates of the molar weight were consistent with that of the monomer of tropoelastin. The value of \(B\) increased with temperature (Table I) indicating molecular expansion and/or asymmetry. A monomer-dimer model equation fit to the data did not significantly improve the fit as judged by a lack of improvement in the sums of squares of the residuals.

**Sedimentation Velocity**—Sedimentation velocity experiments were performed on tropoelastin in PBS at 20 and 37 °C. At 48,000 rpm, coacervate was rapidly sedimented to the base of the solution column. Plots of \(dc(r)/dt\) versus \(s^2\) were fit to a single species model using simultaneous nonlinear least squares regression (19). The residuals to the fit are shown in B and the returned values of molar weight and second virial coefficient, \(B\), are shown in Table I.

37 °C were extrapolated to zero concentration to obtain the values of \(s^{20,0}_{20,0}\) and \(D^{20,0}_{20,0}\) (Fig. 4). By using Equation 4 and the values of \(s^{20,0}_{20,0}\) and \(D^{20,0}_{20,0}\), the calculated molar mass of tropoelastin for both species at both 20 and 37 °C was similar to that for tropoelastin monomer (Table II).

The data were also fit with a “whole boundary” approach using the program Svedberg version 3.16 (32) and confirmed the results obtained from fitting plots of \(dc(r)/dt\) versus \(s^2\). Cleaver simulations (33) of the sedimenting boundaries for two species using similar values of \(s^{20,0}_{20,0}\) and \(D^{20,0}_{20,0}\) to those obtained from fits to the experimental data were also performed. With noise added, the simulated boundaries were fit with a two-species model, and the values of the returned parameters were, within error, the same as the values used to generate the boundaries.

**Pulsed Field-gradient Spin-Echo NMR Experiments**—PGSE NMR experiments were conducted on samples of tropoelastin dissolved in either PBS or pure water. A concentration dependence study (Fig. 5) at 20 °C yielded \(D^{20}_{20,0}\) values for the protein in PBS and in water of \((3.59 \pm 0.01) \times 10^{-11}\) m²/s and \((2.29 \pm 0.03) \times 10^{-11}\) m²/s, respectively.

In the presence of salt at 37 °C, tropoelastin at concentrations above ~1.5 g/liter formed a coacervate that settled to the bottom of the NMR sample tube. For this reason, and because concentrations below ~1.5 g/liter necessitated much longer spectral acquisition times, a concentration dependence study was not conducted in PBS at this temperature. Duplicate experiments were conducted to measure the \(D\) of tropoelastin at 10 g/liter in water at 37 °C, and the weight average \(D_{37,0}\) was calculated to be \((3.11 \pm 0.06) \times 10^{-11}\) m²/s.

**Computer Modeling**—When the signal intensities from Model I (see “Materials and Methods”) were plotted as a function of \(b\) without the addition of noise, a slight systematic deviation from linearity was visually discernible. Once noise was added to the simulated data it became impossible to determine whether or not there was any systematic deviation from linearity even after the regression line was superimposed on the plot. The gradient of the regression line, which corresponded to the diffusion coefficient in a one-species system, was ~4.6 \(\times 10^{-11}\) m²/s.

The data generated by Model II (see “Materials and Methods”) are summarized in Table III. They predicted that the values of \(s^{20,0}_{20,0}\) and \(D^{20,0}_{20,0}\) for a hydrated sphere of tropoelastin monomer would decrease by ~30 and 90% when it was modeled as a flexible chain and a rigid rod, respectively. An inverse relationship of the same order applied to the values of \(f^0_{20,0}\) and \(f^0_{20,0}\). It is notable that the effect of change in the degree of hydration was more significant for the hydrated sphere model than for the other two models.

**Determination of the Critical Concentration of Coacervation**—By using the scintillation proximity assay, the equilibrium established between the soluble species of tropoelastin and the coacervate was not disturbed and was quantifiable. Fig. 6A shows the normalized values of radioactivity (representing tropoelastin in close proximity to the scintillant) plotted against the total concentration of tropoelastin. The data show two approximately linear components with a discontinuity at ~2 g/liter. The data above 2 g/liter were fitted by linear regression. Simple extrapolation of the fit to the abscissa represents an estimate of the critical concentration, \(c_c\), for coacervation of ~2.4 g/liter.

In sedimentation studies performed at 2,000 rpm, the absorbance at 280 nm of the tropoelastin sample at 20 °C was recorded. This absorbance represents the total concentration of tropoelastin since significant coacervation does not occur at this temperature. Upon raising the temperature from 20 to
37 °C, the absorbance at 360 nm increased due to light scattering caused by aggregation of tropoelastin molecules in the solution column. These aggregates sedimented rapidly to the base of the solution column. When the temperature stabilized at 37 °C, the absorbance at 280 nm decreased from its original value at 20 °C and was again recorded. This absorbance represents the concentration of soluble tropoelastin following coacervation. Fig. 6B shows the plot of soluble tropoelastin versus the total concentration of protein. The plot was fitted with a model equation representing the cooperative polymerization of a monomer to a large helical n-mer via a trimeric nucleating species, a situation that approximates that for actin (34, 35).

$C_n = C_1 + aC_0(1 - K_nC_0)$

(eq. 9)

$\sigma = \gamma K_n^2 K_h^2$

(eq. 10)

where $\sigma \ll 1$, $K < K_h$, $C_0$, and $C_1$ are the total concentration and monomer concentration in units of mol/solvent mol, respectively; $\gamma$ is the excess free energy required to deform the linear trimer to give curvature to the helical polymer; $K_h$ is the binding constant for the addition of a monomer to the end of the helical polymer; and $K$ is the binding constant for the addition of a monomer to a simple linear polymer. For this model, the critical concentration equals $1/K_h$. This model fit the data well (solid line, Fig. 6B) and returned a value for the critical concentration of 3.4 ± 0.1 g/liter.

**DISCUSSION**

**Sedimentation Equilibrium—**Our sedimentation equilibrium results showed that the recombinant tropoelastin used in this study could be centrifuged to simultaneous sedimentation and chemical equilibrium at both 20 and 37 °C. The excellent overlap of the plots of the $Q$ function versus $c(r)$ from three different loading concentrations of protein (e.g. Fig. 1B) showed that the recombinant protein was free of soluble, irreversibly
and in pure water (\(20 °C\)). The intercepts of the two lines with the 
vertical axis show the dependence of the signal on concentration in PBS (\(37 °C\), for tropoelastin in PBS) for the linearized data were fit to 
Equation 4. The value of the frictional ratio, \(f_{s,20}^0\), was calculated to be \(4.91 \times 10^{-8}\) g/s.

We estimated a value of the second virial coefficient, \(B\), for tropoelastin using an estimate of its net charge and based on it being a hard incompressible sphere (Table I). This value (\(4.27 \times 10^{-7}\) mol/g\(^2\)) is similar to the experimentally determined value obtained at 20 °C (Table I), indicating that at this temperature tropoelastin molecules are, on average, relatively compact and globular. At 37 °C, however, the experimentally determined value of \(B\) increased significantly (Table I). Assuming that the nonideality due to charge was constant with temperature, these results represent an equivalent spherical radius, \(R_e\), of 3.2 and 7.3 nm at 20 and 37 °C, respectively, in contrast to an \(R_e\) of 2.6 nm for a smooth, compact, spherical particle of tropoelastin (Equation 7). This suggests that, on average, tropoelastin molecules are more expanded or asymmetric at the higher temperature.

**Sedimentation Velocity**—The results from sedimentation equilibrium experiments are consistent with those from sedimentation velocity experiments. Only monomer was observed at both 20 and 37 °C in the sedimenting boundary. Furthermore, at each temperature, two different forms of tropoelastin monomer, Species 1 and 2, existed (Figs. 3 and 4, Table II). The two species were present at approximately equal concentration at both temperatures (data not shown) and did not appear to interconvert significantly over the time scale of these experiments (up to 5 h). If the species interconverted rapidly, then a single symmetrical sedimenting boundary would have been observed that could have been fitted with a single species model returning a molar weight equivalent to the monomer weight of tropoelastin.

The value of the frictional ratio, \(f_{s,20}^0/f_0\), represents the degree of deviation, due to hydration, rugosity, asymmetry, and expansion of the molecule, from the minimum possible value of 1.0 for a hard, incompressible, unhydrated sphere. Compact globular proteins often yield a fractional ratio of about 1.2, the number being greater than 1.0 principally due to hydration. For tropoelastin, Species 1 represents a population of expanded or asymmetric molecules at both 20 and 37 °C; that is, the value of \(f_{s,20}^0/f_0\) is large at \(-2\) (Table II). In contrast, Species 2 which is quite compact and symmetrical at 20 °C becomes somewhat more expanded/asymmetric when the temperature is raised to 37 °C (Table II). This expansion/asymmetry in Species 2 at the higher temperature is consistent with the
Properties of Human Tropoelastin

TABLE III

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hard hydrated sphere</th>
<th>Flexible chain</th>
<th>Rigid rod</th>
</tr>
</thead>
<tbody>
<tr>
<td>( s_0^0 ) (S)</td>
<td>2.8(^a)</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>( D_{20,w}^0 ) ( (10^{-11} \text{m}^2/\text{s}) )</td>
<td>4.05</td>
<td>6.72</td>
<td>5.13</td>
</tr>
<tr>
<td>( f'_{20,w} ) ( (10^{-7} \text{g/s}) )</td>
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<td>0.79</td>
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<tr>
<td>( f_{20,w}/f_0 )</td>
<td>1.22</td>
<td>1.59</td>
<td>1.59</td>
</tr>
</tbody>
</table>

\(^a\) Hydration in H\(_2\)O molecules/residue.

Sedimentation velocity data alone cannot distinguish between expansion and asymmetry associated with a large frictional ratio (36), and additional experimental information is required to unequivocally determine the hydrodynamic properties of tropoelastin. Typically, the concentration dependence of the sedimentation coefficient, \( k_v \), can be used together with the intrinsic viscosity of the protein, \( [\eta] \), to determine the molecular shape, regardless of its size (37). A \( k_v/[\eta] \) value close to 1.6 combined with a large frictional ratio indicates an expanded spherical molecule as, for example, occurs for the tetramer of spectrin (38). Smaller values of \( k_v/[\eta] \) combined with a large frictional ratio suggest greater molecular asymmetry as occurs for myosin \( (k_v/[\eta] = 0.3; \text{see Ref. 37}) \). However, in our sedimentation velocity experiments, each species would have sedimented more slowly in the presence of the other than it would alone (23), thus resulting in abnormally large values of \( k_v \). If Species 1 and 2 could be separately purified, then it may be possible to distinguish the degree to which the individual species are either expanded or asymmetric through measurement of \( k_v/[\eta] \).

Other techniques such as small angle X-ray scattering and electron microscopy could be used to determine the shape of human tropoelastin (36, 39). In electron micrographs, purified bovine and chick tropoelastin appeared spherical with a diameter of 5–7 nm (4, 40). Chick tropoelastin was interpreted as existing as a random coil with an average intrinsic viscosity of 2.1 S as determined by sucrose density gradient centrifugation (41). This value is similar to the \( s_0^0 \) of Species 1 at both 20 and 37°C and together with electron micrographs of chick tropoelastin (4) suggest that Species 1 may adopt an expanded spherical form in solution.

**PGSE NMR**—Through PGSE NMR studies, the diffusion coefficient of tropoelastin in water at 20°C was significantly smaller than in the same medium at 37°C, as was expected. However, the diffusion coefficient in water at 20°C was also significantly less than in PBS at the same temperature. This is likely to be due to conformational differences in tropoelastin in the two media. There could also have been a diminution in the extent of monomer-monomer interactions due to the absence of electrostatic shielding in water resulting in surface charge repulsion. The low signal-to-noise ratio in the \(^1\)H NMR spectra of tropoelastin in PBS at 37°C was due to coacervation of tropoelastin, consistent with the notion that coacervation is predominantly the result of hydrophobic interactions (3, 4).

Estimates of the diffusion coefficient, \( D \), of tropoelastin obtained from sedimentation velocity and PGSE NMR data constitute a novel comparison of data from each method. The hydrodynamic parameters estimated from the sedimentation velocity experiments were biased toward those of the monomeric species since high molecular weight aggregates rapidly sedimented to the base of the centrifuge cell in the presence of a high centrifugal field. On the other hand, the NMR method yielded a tracers, or weight average self-diffusion coefficient which, when extrapolated to the abscissa determined from three independent scintillation proximity assays at 35°C, represented the best fit to the data for a model representing the hydrodynamic parameters estimated from the sedimentation velocity experiments, each species would have sedimented more slowly in the presence of the other than it would alone (23), thus resulting in abnormally large values of \( k_v \). If Species 1 and 2 could be separately purified, then it may be possible to distinguish the degree to which the individual species are either expanded or asymmetric through measurement of \( k_v/[\eta] \).

To support the interpretation of the hydrodynamic parameters determined from the sedimentation velocity experiments, a series of molecular modeling studies was performed to predict hydrodynamic properties of the protein (Table III). A hard sphere of tropoelastin hydrated at 2.8 molecules of water per amino acid residue has the "expected" frictional ratio of \( -1.2 \). This increases modestly to 1.6 upon changing to a flexible chain, whereas a dramatic increase results from a change to a rigid rod conformation (Table III). Therefore, we propose an explanation for the sedimentation data; there exist two species of tropoelastin monomer in solution, one of which, Species 2, displays properties between that of a globular protein and a flexible coil, whereas Species 1 has a more expanded/asymmetric form.

![Figure 6](image-url)

**Fig. 6. Determination of the critical concentration of coacervation.** A, plot of normalized counts versus total concentration determined from three independent scintillation proximity assays at 35°C (C, D, and E). The straight line represents the linear regression of the data above 2 g/liter which, when extrapolated to the abscissa, represents an estimate of the critical concentration for coacervation (2.4 g/liter). B, plot of monomer concentration versus total concentration determined from sedimentation experiments at 37°C (+ and ×). The black line represents the best fit to the data for a model representing the helical polymerization of a monomer to an n-mer via a nucleating trimer. The returned value of the critical concentration for this model was 3.4 ± 0.1 g/liter.
estimated by linear regression analysis. However, the inter-
experimental variation was likely to be such that a deviation of
the straight-line fit from the data would not be statistically
significant.

In contrast to the sedimentation velocity data, the PGSE
NMR data did not provide evidence of two non-interacting
species. Computer modeling (see Model I under “Materials and
Methods” for details) showed that for a system of two non-
interacting species with diffusion coefficients of $~4$ and $~6 \times
10^{-11}$ m$^2$/s, the resolution of the data from a PGSE NMR
experiment would be insufficient to discern a deviation in lin-
earity of the Stejskal-Tanner plot. Additionally, when the con-
centrations of the two species were set as equal in the computer
modeling, the apparent diffusion coefficient calculated by lin-
ear regression favored the slower species. This was due to the
more rapid attenuation of the signal from the faster diffusing
species as the field gradient was increased, such that its overall
relative contribution to the signal intensity was reduced.

**A Model for Coacervation** —There was no evidence to suggest
that either Species 1 or 2 was incompetent. On the basis of their
individual concentrations before and after coacervation,
we both participated in the coacervation process (data not shown).
Furthermore, coacervation is a rapidly reversible process (of
the order of minutes), but the two species interconvert only very
slowly (hours or longer). This suggests the coacervate
consists of a mixture of the two species rather than a conver-
sion of one species to the other which then forms the coacerv-
ate. Further work is required to determine the stoichiometry
of the two species in the coacervate with good accuracy
and precision.

The process of coacervation was further examined by scintil-
lation proximity assays. The biphasic plot obtained at 35°C
(Fig. 6A) is typical of the helical polymerization of macromole-
cules as diverse as actin, insulin, fibrin, and tropocollagen (43).
Helical polymerization occurs above the critical concentration
because of the presence of unstable, slow forming nuclei that
act as seeds for the rapid production of filaments. The system
reaches equilibrium or a steady state when the monomer con-
centration falls to the critical concentration (35). We could not
detect nuclei under the sedimentation velocity conditions used,
but time course measurements of tropoelastin coacervation
have demonstrated an initial lag phase prior to a sigmoidal rise
in turbidity (1, 14), consistent with the nucleated condensation
mechanism of helical polymerization (44).

Turbidity measurements may not provide additional infor-
mation about the coacervation process since turbidity is not
proportional to the mass of coacervated tropoelastin.3 Indeed,
the increase in turbidity is due to the formation of microscopic
oil-like droplets, or microcoacervates, as the solution is heated
(45). However, on the basis of model fitting to absorbance
measurements for soluble tropoelastin (Fig. 6B), trimeric tro-
poelastin may be a nucleating species for coacervation although
the true situation, as with actin, is likely to be more complex (35).

In summary, we have shown that monomers are the predom-
inant form of soluble tropoelastin before and during coacerva-
tion. These monomers appear to exist in solution in more than
one conformation. Coacervation of tropoelastin proceeds via the
cooperative association of tropoelastin monomers to form large
aggregates, in a process that is the same as or similar to the
formation of helical polymer. It would be useful to demonstrate
directly the presence of oligomeric intermediates under condi-
tions different from those used in the present experiments.
Although we have not separated the two species of tropoelastin

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3 S. A. Jensen and A. S. Weiss, unpublished data.

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**REFERENCES**