Frequency Dependence of the Shear Moduli of Spectrin Studied Using a Multiple Lumped Resonator Viscoelastometer

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The frequency dependence (119–7860 Hz) of the storage and loss shear moduli, G' and G'', of human erythrocyte spectrin dimer crude solutions at 22.5 °C has been measured using a Birnboim–Schrag multiple lumped resonator viscoelastometer. The measurements were carried out on solutions of ionic strength 1 mM containing 1.1–3.7 mg ml^{-1} spectrin. This corresponds to the terminal zone for G' and G''. Analysis of the data using the standard theory of hybrid relaxation spectra yields a relaxation time of 22.5 ± 1 μs. The pure spectrin dimer relaxation time is estimated to be 16 ± 3 μs. This result suggests that at an ionic strength of 1 mM, the spectrin dimers are extended and that the main relaxation process is simple end-over-end rotation.

Spectrin is a protein believed to play an important role in determining the mechanical properties of the erythrocyte membrane.[1,2] The erythrocyte shape is generated by the so-called membrane skeleton of which spectrin is the major component. An understanding of spectrin's dynamic properties is expected to be important for the detailed elucidation of how the molecule plays its functional role.

The protein is composed of two different polypeptides (monomers) of molecular weight 220 and 240 kg mol^{-1}. In the membrane skeleton spectrin is found as heterodimers, tetramers or higher-order oligomers. Electron microscopic data show that the spectrin monomer is an elongated worm-like molecule that has a contour length of about 100 nm.[3] The heterodimer is formed of two monomers interconnected at each end. Light scattering[4,5] and intrinsic viscosity data[6,7] show that the spectrin dimer resembles a random coil at physiological ionic strength, but corresponds more and more to a stiff rod as the ionic strength is decreased. Birefringence relaxation studies of spectrin dimer[8,9] at an ionic strength of 1–5 mM and 20 °C yield a major relaxation time of about 2–4 μs, and measurements on spectrin tetramer[10,11] yielded the same value. Both intrinsic viscosity data and theoretical analysis, assuming that spectrin dimers are stiff rods with of about 100 nm length, yield a relaxation time of about 16 μs under these conditions. The reason for this discrepancy is not known.

In an attempt to resolve this inconsistency we report here on a viscoelastic study of spectrin dimer solution. The theory of polymer solution viscoelastic properties is well established, and analysis of the data yields characteristic relaxation times for the polymer.[12] We have studied the dimers' viscoelastic properties (shear storage and loss moduli) in the frequency range 119–7860 Hz using a Birnboim–Schrag multiple lumped resonator.[13] The viscoelastic data for spectrin presented here are consitent with the light scattering and intrinsic viscosity data, and suggest that spectrin dimers behave as stiff rods at an ionic strength of 1 mM.

Theory

The principal quantities that can be obtained from the measured complex shear moduli extrapolated to infinite dilution are the intrinsic viscosity, the rotational relaxation time, and the longest relaxation time for flexional motions. It is convenient to use the reduced intrinsic storage and loss shear moduli defined as in eqns. (1) and (2) where M is the

$$[G']_R \equiv (M/RT) \lim_{c \to 0} (G'/c) \quad (1)$$

$$[G'']_R \equiv (M/RT) \lim_{c \to 0} \{(G'' - \omega\eta_S)/c\} \quad (2)$$

molecular weight, R the gas constant, T the absolute temperature, c the polymer concentration, G' and G'' the storage and loss shear moduli, ω the radian frequency of oscillation and η_S the solvent viscosity. The reduced intrinsic shear moduli have dimensions of unity.

The intrinsic viscosity, $[\eta]$, is related to the reduced intrinsic loss shear modulus at low frequencies as shown in eqn. (3).

$$[\eta] = (RT/M) \lim_{\omega \to 0} \frac{[G'']_R}{\omega\eta_S} \quad (3)$$

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Partially flexible elongated molecules are commonly modelled as semiflexible rods with the frequency dependence of the intrinsic shear moduli as expressed by the empirical hybrid relaxation spectra[14] in eqns. (4) and (5).

$$[G']_R = \frac{m_1\omega^2\tau_0^2}{(1 + \omega^2\tau_0^2)} + zZ'(\omega\tau_1) \quad (4)$$

$$[G'']_R = \omega\tau_0 \left[\frac{m_1}{(1 + \omega^2\tau_0^2)} + m_2\right] + zZ''(\omega\tau_1) \quad (5)$$

where τ_0 is the main rotational relaxation time and $Z'(\omega\tau_1)$ and $Z''(\omega\tau_1)$ are the reduced moduli of the Zimm theory for random coils for which τ_1 is the longest relaxation time. The values of the parameters m_1, m_2 and z and the ratio τ_0/τ_1 depend on the details of the molecular theory used.[12] The validity of the hybrid theory has been verified in several studies of elongated biopolymers.[14–19]

Materials and methods

Isolation of spectrin dimers. Human erythrocyte ghosts were prepared from 300–400 ml human blood collected in Fenwal JF 15 bags (Travenol Laboratories, S. A. Belgium) containing 63 ml citrate phosphate dextrose adenine anticoagulant solution [327 mg citric acid monohydrate, 2.63 g sodium phosphate monohydrate, 2.90 g dextrose (anhydrous) and 27.5 mg adenine per 100 ml]. The preparation was started 3–5 weeks after the blood had been drawn from healthy adults. Unless otherwise stated, all preparative steps were carried out at pH 7.6 and 4 °C.

Human erythrocyte ghosts were prepared according to Stokke *et al.*[20] The erythrocytes from two Fenwal JF 15 bags were washed twice in 1000 ml of 310 mosM phosphate-buffered saline and once in 1000 ml of 310 mosM phosphate buffer and centrifuged at 3500 × *g* (6000 rev. min^{-1}, Beckman JA 14 rotor) for 15 min at the end of each wash. The buffy coat and the hard pellet underneath the packed erythrocytes were removed after each centrifugation. The washed cells were hemolysed by diluting the cells up to 1400 ml with 20 mosM phosphate buffer. The suspension was stirred for 30 min. The erythrocyte ghosts were then separated from hemoglobin by means of a plasma separator (Plasmaflo AO-OSH, Asahi Medical Co., Ltd, Tokyo, Japan).[20] Within 2–3 h, 600–700 ml pink to white ghosts were achieved. The erythrocyte ghosts where then packed by centrifugation at 19000 × *g* (14000 rev. min^{-1}, Beckman JA 14 rotor) for 45 min.

Packed ghosts from two bags of blood were diluted to a final volume of 1500 ml in 1 mM Tris/0.1 mM EGTA/0.05 mM dithiothreitol/0.02 mg ml^{-1} phenylmethanesulfonyl fluoride using buffer pre-equilibrated to 37 °C. Spectrin and actin were extracted by incubation at 37 °C for 30 min. The spectrin–actin depleted vesicles were removed by pelleting at 19500 × *g* (14000 rev. min^{-1}, Beckman JA 14 rotor) for 45 min. The supernatant was saved and the proteins were precipitated for 2 h by the addition of ammonium sulfate to a concentration of 29 g per 100 ml. The precipitated proteins were recovered by centrifugation at 14500 × *g* (12000 rev. min^{-1}, Beckman JA 14 rotor) for 15 min, and the pellet dialysed against 4 × 1000 ml of 1 mM Tris/0.1 mM EGTA/0.05 mM dithiothreitol for 24 h. To enhance the conversion of spectrin oligomers to dimers, the dialysate was treated with 0.02 mg ml^{-1} phenylmethanesulfonyl fluoride and then heated to 37 °C for 30 min. The solution was then centrifuged at 12800 × *g* (45000 rev. min^{-1}, Beckman Ti 50 rotor) for 60 min. The supernatant was dialysed against 4 × 1000 ml 1 mM Tris/0.1 mm EGTA/0.05 mM dithiothreitol/0.02 % sodium azide for 24 h. The spectrin concentration was determined by measuring the absorbance at 280 nm using a specific absorbance of A (1 cm, 1 %, 280 nm) = 10.1[4] for spectrin. Two bags of blood yielded about 160 mg of spectrin.

Viscoelastic measurements. The storage and loss shear moduli, G' and G'', were measured using the new twin Birnboim–Schrag multiple lumped resonator device built in our laboratory[13] after the concept of Schrag and Johnson.[21] The apparatus has a computerized data acquisition and processing system. All measurements were done at 22.5 °C. The temperature stability of the instrument is ± 0.001 °C. The apparatus consists of two titanium resonators mounted in separate titanium houses. The resonators exhibit a total of 10 resonance frequencies in the frequency range 119–7860 Hz. We followed the filling and cleaning procedure described by Hvidt *et al.*[14] The solvent viscosity, η_S, was measured in the MLR apparatus on the dialysate.

Estimation of spectrin relaxation times. The reduced intrinsic storage and loss shear moduli, $[G']_R$ and $[G'']_R$ are in general obtained from plots of $(G'M/cRT)^{1/2}$ and $(G'' - \omega\eta_s)M/cRT$ versus concentration at each frequency, by extrapolation to zero concentration.[22] Such plots in the range 1.1–3.7 mg ml^{-1} [13] (data not show here) depict no significant dependence on concentration at any frequency. We have therefore used average values of G' and G'' for each frequency to obtain estimates for $[G']_R$ and $[G'']_R$ by eqns. (1) and (2). An additional argument for the use of averaged values is that for all our spectrin dimer solutions $[\eta]c \approx 0.1$–0.3. This means that in the solutions the spectrin–spectrin interaction is negligible.

The spectrin relaxation times were estimated by minimizing the sum of squared residuals (SSR) of the experimental and theoretical reduced intrinsic loss moduli ($[G']_{EXP}$, $[G'']_{EXP}$ $[G']_{THEOR}$ and $[G'']_{THEOR}$). The reduced intrinsic theoretical loss moduli were obtained from eqns. (4) and (5) using $m_1 = 0.6$ and $z = 1.0$. The value of m_2 may be constant or may be a parameter in the minimizing procedure, varied within the range 0.25–0.40. We used the curve-fitting algorithm described by Nelder and Mead[23] and Cacesi and Cacheris[24] (Simplex algorithm). The parameter estimation was based on the experimental $[G']_R$ and/or $[G'']_R$ data and using a weight σ_i = 1/(standard deviation)

$$SSR = \sum_{i=1}^{n\text{data}} \{\sigma_i \cdot ([G]_{THEOR} - [G]_{EXP})\}^2, \quad (6)$$

for each datapoint i for $[G']_R$ or $[G'']_R$, respectively. The parameter estimation was based either on data of $[G']_R$ or $[G'']_R$ alone, as expressed in eqn. (6), or on both $[G']_R$ and $[G'']_R$ simultanously, as expressed in eqn. (7) where n and m are the number of data points of $[G']_R$ or $[G'']_R$, respectively.

$$SSR = \sum_{i=1}^{n} \{\sigma_i \cdot ([G']_{THEOR} - [G']_{EXP})\}^2 + \sum_{j=1}^{m} \{\sigma_j \cdot ([G'']_{THEOR} - [G'']_{EXP})\}^2 \quad (7)$$

In our computer program the number of relaxation times to be estimated is selectable. The theoretical curve using the estimated values of the relaxation times and the m_2 value is shown graphically together with the experimental data points of the intrinsic values of G' and G''. The computer program is written using IBM Professional FORTRAN.

Results and discussion

The elution profile of the crude spectrin extract on a Sepharose Cl–4B gel filtration column is shown in Fig. 1. From Fig. 1 the relative amounts of spectrin dimers, tetramers and higher-order oligomers/aggregates in the crude extract is estimated to be 60, 20 and 20 %, respectively. The influence of tetramers on the total end-over-end rotational time is discussed later in this text. The higher-order oligomers/aggregates are assumed to be even more elongated than the tetramers at this low ionic strength, and their influence on the total end-over-end rotational time is therefore neglected.

Plots of the reduced intrinsic storage and loss moduli versus reduced frequency are shown in Fig. 2. The data correspond to the terminal zone of spectrin dimers, that is $\omega\tau_0 < 1$ and the experimental data can therefore only be used to estimate τ_0 and none of the higher-order relaxation

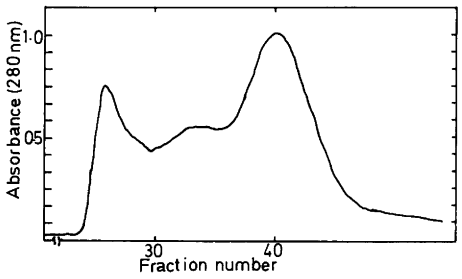

Fig. 1. Elution profile of the crude spectrin extract on Sepharose Cl-4B gel filtration column.

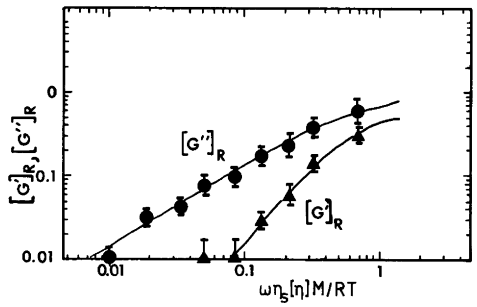

Fig. 2. Reduced intrinsic storage and loss shear moduli, $[G']_R$ and $[G'']_R$ versus reduced frequency, $\omega\eta_S[\eta]M/RT$ for crude spectrin dimer (intrinsic viscosity $[\eta]$ = 78 ml g^{-1} and M = 460 kg mol^{-1} for spectrin dimer). Data obtained using the twin Birnboim–Schrag multiple lumped resonator at 22.5 °C, in low salt solution and pH 7.5. The solid lines drawn are the theoretical curves [eqns. (4) and (5)] for τ_0 = 22.5 μs, m_2 = 0.25.

times. The end-over-end rotational relaxation time estimated for crude spectrin is 22.5 ± 1 μs.

The frequency dependence of the theoretical intrinsic shear moduli as presented in this section is limited to uniform molecular weights of the sample. The MLR instrument requires ca. 47 ml of about 2–3 mg ml^{-1} spectrin solutions to carry out the viscoelastic measurements. That corresponds to a total of about 100–130 mg spectrin. Standard purification of the spectrin crude extract on a Sepharose Cl-4B gel filtration column results in a substantial dilution and reduction in the spectrin yield. One standard bag of 450 ml blood yields about 30 mg of pure spectrin dimer at a concentration of about 0.6 mg ml^{-1}. Crude spectrin extract was therefore used in the present study, despite purified spectrin dimer solutions clearly being preferable.

In the parameter estimation we have assumed that the solution consisted of pure spectrin dimers. To estimate the influence on the calculated end-over-end rotational time of the tetramers present in the solution, we carried out a parameter estimation on simulated data from a solution containing 25 % tetramer. The contribution from tetramers and dimers to G' and G'' for the solution is calculated from eqns. (1), (2), (4) and (5) using M = 460 kg mol^{-1}, L = 100 nm, τ_0 = 15 μs for the dimer and M = 960 kg mol^{-1}, L = 200 nm, τ_0 = 120 μs for the tetramer. Only the longest rotational relaxation time was taken into consideration, then the total shear storage modulus $G' = G'_{dimer} + G'_{tetramer}$ and loss modulus $G'' = G''_{dimer} + G''_{tetramer}$ were calculated. The reduced intrinsic moduli are then found according to eqns. (1) and (2) using M = 460 kg mol^{-1} and concentration equal to the sum of the dimer and tetramer concentration. The intrinsic storage and loss shear moduli were calculated for 10 frequencies corresponding to the frequency range of the MLR. The values obtained were analysed in the same way as the experimental data. The end-over-end rotational time was estimated to be 21 μs. This is 40 % higher than the value of 15 μs for the pure dimer solution. This suggests that our result of τ_0 = 22.5 ±

1 μs for the crude spectrin extract indicates a value of τ_0 = 16 ± 3 μs for pure spectrin dimers, when the tetramer is assumed to add 30–60 % to the apparent dimer end-over-end rotational time.

The above value of τ_0 for pure spectrin dimers yields an intrinsic viscosity in 1 mM Tris pH 7.5 of $[\eta]$ = 71 ± 15 ml g^{-1} [eqn. (3)]. Stokke and Elgsaeter[7] reported a value of $[\eta]$ = 78 ± 8 ml g^{-1} for purified spectrin dimers under similar conditions (2 mM NaCl) using a Cartesian diver low-shear viscometer. In the same viscometric study it is reported that spectrin in low ionic strength resembles a rigid rod with an axial ratio of 31 and a contour length of about 110 nm. Our MLR-data thus agree well with the earlier intrinsic viscosity data.

Conclusions

The new independent information yielded by this study is the frequency dependence of the spectrin dimer storage modulus. $[G'']_R$ and $[G']_R$ both yield τ_0 = 22.5 ± 1 μs for the crude spectrin extract, and the estimated τ_0 for spectrin dimer is τ_0 = 16 ± 3 μs. The expected value[25] of the end-over-end rotational time is 15 μs when spectrin dimer is assumed to be a thin, 100 nm long rod. This MLR study therefore supports the model that spectrin dimers in 1 mM Tris behave as extended rods. Earlier birefringence relaxation data[8–11] yielded values of $\tau_0 \approx$ 2–4 μs in 0.5–5 mM salt concentration. The reason for this discrepancy is not known at present, but probably involves conformational changes in spectrin introduced by the electrical field used to orientate the spectrin dimers in the birefringence relaxation experiment. The present data agree well with viscometric data[7] for spectrin dimers in 2 mM NaCl solution.

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