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Human erythrocyte spectrin dimer intrinsic viscosity: Temperature dependence and implications for the molecular basis of the erythrocyte membrane free energy

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We have determined experimentally the temperature dependence of human erythrocyte spectrin dimer intrinsic viscosity at shear rates 8–12 s^{-1} using a Cartesian diver viscometer. We find that the intrinsic viscosity decreases from 43 ± 3 ml/g at 4°C to 34 ± 3 ml/g when the temperature is increased to 38°C. Our results show that spectrin dimers are flexible worm-like macromolecules with persistence length about 20 nm and that the mean square end-to-end distance for this worm-like macromolecule decreases when the temperature is increased. This implies that the spectrin dimer internal energy decreases when the end-to-end distance is increased and that the free energy increase associated with making the end-to-end distance longer than the equilibrium value for the free molecules is of entropic origin. The temperature dependence of the erythrocyte membrane shear modulus reported previously in the literature therefore appears mainly to be due to temperature dependent alterations in the membrane skeleton topology.

Introduction

Erythrocytes are subjected repeatedly to large deformations when blood is circulated through the cardiovascular system. Normal red cell strength and deformability are therefore crucial in order to maintain the erythrocyte integrity. Because mammalian erythrocytes have no transcellular cytoskeleton, it is widely recognized that the elastic properties and shape of these cells are manifestations of the supramolecular structure of their cell membranes. The cell membrane lipid bilayer alone is not able to sustain the shear stress imposed on the red cell during the blood circulation. It is therefore generally acknowledged that the erythrocyte membrane skeleton plays a crucial role in determining the elastic properties of the erythrocyte membrane.

The erythrocyte membrane skeleton consists of a continuous locally two-dimensional protein network spanning the entire cytoplasmic surface of the membrane lipid bilayer. The main components of this network are spectrin, actin, and proteins 4.1 and 4.9 [1–4]. Spectrin constitutes about 75% of the mass of the erythrocyte membrane skeleton [5]. Spectrin is a water soluble, highly flexible, and elongated, but not a random coil type protein [6–15]. The amino acid sequence of spectrin indicates that the spectrin α- and β-chains consist of 20 and 18 interconnected structurally nearly identical segments, respectively [16]. Each segment is believed to contain three short α-helices and to be rather stiff, but electron microscopic studies [7] indicate that the spectrin α- and β-chains are worm-like rather than kinked. In the intact erythrocyte membrane skeleton the spectrin molecules are linked into a network by specific non-covalent associations between the tail ends of the spectrin tetramers/oligomers and protein 4.1 and actin oligomers [4,17]. There is no indication of any significant non-specific chain side-by-side association of the kind commonly found in many

other non-covalently cross-linked biopolymer gels. The mechanical properties of the erythrocyte membrane have been studied extensively during the last 10–15 years [18–20]. Because of the fluid nature of the membrane lipid bilayer, the membrane skeleton must be the origin of the increase in elastic free energy associated with membrane shear deformation. The molecular details of how the various components of the membrane skeleton contribute to this increase in deformation free energy is unknown, but because spectrin by far is the major component of the membrane skeleton, this molecule is likely to contribute most to the change in free energy associated with shear deformation. Based on thermoelastic measurement of the erythrocyte membrane Waugh and Evans [21] came to the conclusion that deforming the membrane at constant area was associated with an increase in spectrin entropy and internal energy.

In this paper we report on an experimental determination of the temperature dependence of human erythrocyte spectrin dimer intrinsic viscosity. Our findings lead to a conclusion different from that of Waugh and Evans [21] and may therefore have important implications for the understanding of the molecular basis of the erythrocyte membrane elastic free energy.

Theory

Intrinsic viscosity data of long macromolecules can be used to estimate their flexibility and extension. Yamakawa and Yoshizaki [22] showed theoretically that the intrinsic viscosity of a flexible worm-like Porod-Kratky macromolecule with total contour length L, diameter d, and persistence length q, can be expressed as

$$[\eta] = [\eta]_R \cdot f(L,d,q) \tag{1}$$

where $[\eta]_R$ is the intrinsic viscosity of a stiff rod with length L and diameter d (when $L/q > 2.278$), or the intrinsic viscosity of a random coil molecule (when $L/q < 2.278$). The mathematical expression for $f(L,d,q)$ is given by Yamakawa and Yoshizaki [22]. When L and d are known, Eqn. 1 can thus be used to estimate the persistence length q for such a worm-like macromolecule provided that the intrinsic viscosity has been determined experimentally. For Porod-Kratky chains $q = Z/kT$ where Z is the chain bending force constant, k is the Boltzmann constant and T is the absolute temperature [23]. Because $f(L,d,q)$ decreases with decreasing q for constant L and d the theory predicts that both the persistence length and the intrinsic viscosity of Porod-Kratky chains decreases with increasing temperature. Flory [24] showed that the unperturbed mean square end-to-end distance of Porod-Kratky chains equals:

$$\langle r^2 \rangle_0 = 2Lq(1-(q/L)(1-\exp(-L/q))) \tag{2}$$

Yamakawa and Yoshizaki [25] showed that $\partial \ln\langle r^2 \rangle_0 / \partial T = -1/T$.

The Flory-Fox theory [26] yields that for randomly coiled chains:

$$[\eta] = \phi_c \cdot \langle r^2 \rangle^{3/2}/M = \phi_c \cdot \langle r^2 \rangle_0^{3/2} \alpha^3 / M \tag{3}$$

where ϕ_c is a molecule independent constant, $\langle r^2 \rangle$ is the mean square end-to-end distance, M is the molecular weight, and α is the chain expansion due to excluded volume effects. Bloomfield and Zimm [27] showed theoretically that the intrinsic viscosity also of extended non-Gaussian chains can be expressed by Eqn. 3, but then ϕ_c is a function of chain flexibility. Together, Eqns. 2 and 3 can be used to obtain an additional estimate of the persistence length for a Porod-Kratky macromolecule provided $[\eta]$, ϕ_c and L for the macromolecule are known.

Swollen networks of flexible, elongated and charged macromolecules are commonly termed ionic gels [28–30]. The elastic shear modulus, G, of a locally two-dimensional ionic gel, is given by [19,31]:

$$G = N_c kT \cdot \langle r^2 \rangle / \langle r^2 \rangle_0 \tag{4}$$

where N_c is the number of macromolecules per unit area in the network undergoing independent thermal motions, and $\langle r^2 \rangle$ and $\langle r^2 \rangle_0$ are the mean square end-to-end distance of the macromolecules when they are part of the macromolecular network and not subjected to the restrictions of the network, respectively. It is important to note that this theoretical expression for the shear mod-

ulus rests only on the assumption that the end-to-end distance of the macromolecules making up the gel follows Gaussian statistics and not on the assumption that the gel is made up of random coil type macromolecules [31]. For most extended flexible macro-molecules, the end-to-end distance follows approximately Gaussian statistics even though they are not truly random-coil macromolecules [32]. The temperature dependence of the surface elastic shear modulus is given by:

$$(T/G)(\partial G/\partial T)_{\lambda,\alpha} = 1 + (T/N_c)(\partial N_c/\partial T) - T(\partial \ln\langle r^2\rangle_0/\partial T) \quad (5)$$

where λ is the shear deformation and α is the isotropic expansion. Note that $(T/G)(\partial G/\partial T)_{\lambda,\alpha}$ may be negative as well as positive.

Materials and Methods

Preparation of human erythrocyte ghosts

Ghosts were either 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), or from packed red blood cells in phosphate buffered saline with adenine, glucose and mannitol (887 mg NaCl, 16.9 mg adenine, 819 mg glucose and 525 mg mannitol per 100 ml). The preparation started 2–5 weeks after the blood had been drawn from healthy adults. Unless otherwise stated, all preparation steps were carried out at pH 7.6 and 4°C.

The erythrocytes from one Fenwal JF 15 bag 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 5000 × *g* (5000 rev./min, Beckman JA 14 rotor) for 15 min at the end of each wash. Care was taken to remove the buffy coat and other material in the hard pellet underneath the packed erythrocytes after centrifugation. Erythrocytes from one Fenwal JF 15 bag were hemolyzed by adding the washed cells to 1300 ml of 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). The ghost/hemoglobin suspension was recirculated at a flow of 120 ml/min and the hemoglobin solution withdrawn at a rate of approx. 20 ml/min. The ghost/hemoglobin suspension is kept at a constant volume of 600–700 ml in a sealed bottle by automatic refilling of 20 mosM phosphate buffer from a large reservoir. This procedure yielded pink to white ghosts from one Fenwal bag within 2–3 h, and was substantially less laborious than the commonly used washing procedure involving repeated washing by centrifugation [6]. The erythrocyte ghosts were then packed by centrifugation at 19500 × *g* (14000 rev./min, Beckman JA 14 rotor) for 45 min.

Isolation of spectrin dimers

Packed ghosts from one bag 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} phenylmethylsulfonylfluoride using buffer pre-equilibrated to 37°C. Spectrin and actin were then extracted by incubation at 37°C for 30 min. This extraction procedure removed about 80–90% of total ghost spectrin and actin. The spectrin-actin depleted vesicles were removed by pelleting at 19500 × *g* (14000 rev./min, Beckman JA 14 rotor) for 45 min. The supernatant was saved and the proteins were precipitated for 2–3 h by adding ammonium sulphate to a concentration of 29 g/100 ml. The precipitated proteins were recovered by centrifugation at 14500 × *g* (12000 rev./min, Beckman JA 14 rotor) for 15 min, and the pellet dialyzed against 2 × 1000 ml of 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol for 24 h. The solution was then centrifuged at 128000 × *g* (45000 rev./min, Beckman Ti 50 rotor) for 60 min to remove any larger aggregates, and subsequently applied to a Sepharose CL-4B gel filtration column (900 × 36 mm) equilibrated in 130 mM KCl/20 mM NaCl/5 mM Tris/5 mM sodium phosphate/0.1 mM EGTA/0.05 mM dithiothreitol. The column was eluted with the same buffer at a flow rate of 15–20 ml/h and 6-ml fractions collected. The proteins in the effluent were monitored by measuring the ab-

sorbance at 280 nm, and the spectrin dimer fractions pooled. This isolation procedure yielded 20–26 ml of 0.6 mg/ml purified spectrin dimers.

Concentration and purity of spectrin

The spectrin dimer concentration was determined by measuring the absorbance at 280 nm and using specific absorbance A (1 cm, 1%, 280 nm) = 10.1 [6]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out in 0.2% sodium dodecyl sulphate in a 5% stacker/9% separation slab gel essentially according to Laemmli [33]. Polyacrylamide gels were stained with Coomassie blue and routinely dried between two sheets of uncoated cellophane. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was done on samples from most fractions from the gel filtration column of all the spectrin preparations to test the purity of the various fractions. The same analysis was also done on samples from the final spectrin solutions taken both before and after the viscometric measurements.

Cartesian diver viscometry

The pooled spectrin fractions from the gel filtration column were dialyzed against 3×1000 ml of 10 mM KCl/1 mM Tris/1 mM phosphate buffer/0.1 mM EGTA/0.05 mM dithiothreitol/0.020 mg $\cdot$ ml^{-1} phenylmethylsulfonylfluoride (pH 7.5) for 24 h and centrifuged at $128\,000 \times g$ (45 000 rev./min, Beckman Ti 50 rotor) for 60 min. Low shear viscometry was carried out using the Cartesian diver viscometer described by Stokke and Elgsaeter [11]. This instrument has since been improved substantially [34]. The stainless steeel in the metal tube of the diver was replaced with gold covered copper to minimize magnetic hysteresis effects when the external torque was changed. The external torque was generated by a rotating magnetic field using currents 90° out of phase in the two pairs of magnets generating this field. This current was controlled by a digital frequency synthesizer and was reproducible within $\pm 0.2\%$. The optical system for measuring the rotation of the diver was redesigned essentially according to Troll et al. [35]. The rotor chamber was originally surrounded by a water jacket connected to a 10 litre thermostatically controlled water bath (Haake NK 22) [11]. To further reduce temperature fluctuations, a 25 litre isolated buffer tank was included in the temperature regulating part of the viscometer. The specimen temperature was now maintained within ± 0.02 deg. C. The specific viscosity was obtained by measuring the time per revolution, t_0 and t_1, when the Cartesian diver was placed in the solvent and the spectrin solution respectively, and using the relation $\eta_{sp} = (t_1/t_0) - 1$ [36]. The time per revolution was calculated measuring the total rotation time of approx. 200 revolutions of the diver after its initial hydrodynamic stabilization. The specific viscosity was measured at 3–5 different concentrations for each sample. Each sample was only used for viscometry at one temperature. The intrinsic viscosity was obtained as the slope of the specific viscosity versus concentration. Measurements on two or three independent preparations were carried out to determine the intrinsic viscosity at each temperature.

Results and Discussion

A typical spectrin elution profile from the Sepharose CL-4B gel filtration column together with Coomassie blue stained sodium dodecyl polyacrylamide gels of samples from the three major peaks are shown in Fig. 1. The elution profile of low ionic strength cold-extracts from ghosts [6], commonly used to isolate spectrin tetramers, is included in Fig. 1 to illustrate that spectrin tetramers and dimers are well separated by the gel filtration system used. The center of peak III (Fig. 1) contains spectrin dimers and only very small amounts of spectrin tetramers. The sodium dodecyl polyacrylamide gels show that there are no detectable contaminating proteins present in the spectrin samples.

In a viscosity study of spectrin dimer solutions at various temperatures it is important to make certain that there has been no significant conversion from dimers to tetramers. Spectrin dimers, tetramers and higher oligomers are believed to be interconverted through simple thermodynamic equilibria [37–39]. Below 15°C both the dimeric and tetrameric states are kinetically trapped, and there is essentially no interconversion between dimers, tetramers and higher oligomers. However, above 20°C interconversion takes place fairly

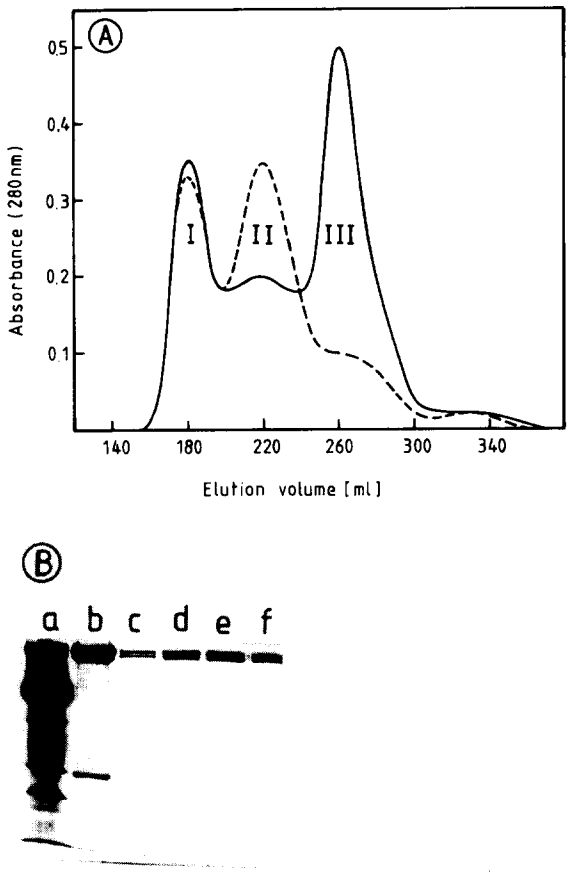

Fig. 1. Typical elution profiles from the Sepharose CL-4B gel filtration column used to separate the various spectrin species when the spectrin dimer preparation procedure (solid line) and the spectrin tetramer preparation procedure (broken line) were used (A). Coomassie blue sodium dodecyl gel electrophoresis of samples from the major peaks in the elution profile is shown in (B). Peak I (lane b) contains both spectrin and actin whereas peaks II (lanes c and d) and III (lanes e and f) contain only spectrin and no detectable amounts of other proteins.

rapidly and the thermodynamic equilibrium is reached within one hour or less. It is therefore important to calculate the extent of such conversion and its effect on the measured viscosity of the spectrin solution.

Below spectrin concentrations of 1 mg/ml the relative amount of spectrin dimers and tetramers can be estimated by considering the equilibrium between spectrin dimers and tetramers only:

$$2[S_2] \leftrightarrow [S_4] \tag{6}$$

where $[S_2]$ and $[S_4]$ are molar concentrations of spectrin dimers and tetramers respectively. The dimer-tetramer association constant K_a:

$$K_a = K_a(I,T) = [S_4]/[S_2]^2 \tag{7}$$

is a function of both ionic strength, I, and temperature, T [37]. Mass conservation requires:

$$[S_{20}] = [S_2] + 2[S_4] \tag{8}$$

where $[S_{20}]$ is the dimer concentration when all the spectrin is present as dimers. Eqns. 7 and 8 yield:

$$[S_2]/[S_{20}] = \left(\sqrt{(1+8K_a[S_{20}])} - 1\right)/4K_a[S_{20}] \tag{9}$$

Fig. 2 shows $[S_2]/[S_{20}]$ as a function of $\log(K_a[S_{20}])$. In the present study we used an ionic strength of 10 mM partly to take advantage of the fact that K_a is substantially lower at this ionic strength than in isotonic salt solutions [37]. The amount of spectrin tetramer in the spectrin dimer sample at 30°C is estimated to be about 6% when the total spectrin concentration is 0.6 mg/ml assuming $M = 460\,000$ and $K_a = 3 \cdot 10^{-4}$ M^{-1} at $I = 0.01$ (Fig. 2). This value of K_a is estimated from the data of Ungewickell and Gratzer [37] assuming the same ionic strength dependence at 30°C as reported at 37°C. We estimate that the partial conversion of spectrin from dimers to tetramers leads to an increase in specific viscosity, η_{sp}, of only about 3% at 30°C when it is assumed that

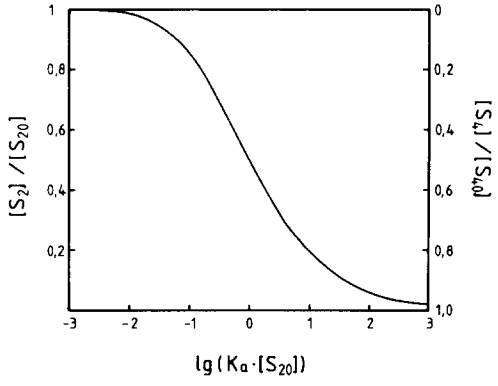

Fig. 2. The fraction of spectrin present as dimers, left scale, and tetramers, right scale, as function of $\log(K_a[S_{20}])$. K_a is the spectrin dimer-tetramer association constant and $[S_{20}]$ is the molar concentration when spectrin is present as dimers only.

the intrinsic viscosity of the spectrin tetramer is twice that of spectrin dimers [11,12,40]. By increasing the temperature to 37°C, the dimer will become more favoured relative to the tetramer. This worst case example (0.6 mg/ml spectrin at 30°C), shows that the systematic error in η_{sp} due to dimer to tetramer conversion for all temperatures and spectrin concentrations used in this study, is substantially smaller than the experimental error (Fig. 3A). Fig. 3A shows the specific viscosity versus spectrin dimer concentration at 4°C and 38°C. Coomassie blue stained sodium dodecyl polyacrylamide gels of the spectrin sample before and after viscometry at 38°C show no significant proteolytic degradation of spectrin (data not shown). The intrinsic viscosity of spectrin dimers in 10 mM KCl at pH 7.5 versus temperature from 4°C to 38°C is shown in Fig. 3B. The intrinsic viscosity of spectrin dimers in 130 mM KCl at pH 7.5 was found to exhibit the same temperature dependence (data not shown).

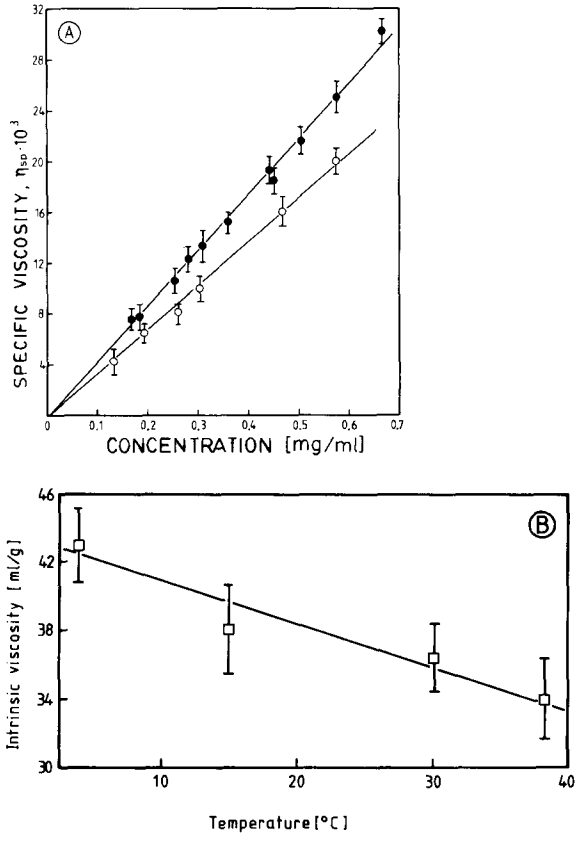

Fig. 3. The specific viscosity of spectrin dimers in 10 mM KCl/1 mM Tris/1 mM phosphate buffer/0.1 mM EGTA/0.05 mM dithiothreitol/0.020 mg · ml^{-1} phenylmethylsulfonylfluoride (pH 7.5) versus spectrin concentration at 4°C (●) and 38°C (○) (A). The intrinsic viscosity of spectrin dimers in 10 mM KCl/1 mM Tris/1 mM phosphate buffer/0.1 mM EGTA/0.05 mM dithiothreitol/0.020 mg·ml^{-1} phenylmethylsulfonylfluoride (pH 7.5) versus temperature (B). The viscometry was carried out at shear rates in the range 8–12 s^{-1}.

An estimate of the root mean square end-to-end distance of spectrin can be obtained from the intrinsic viscosity data using Eqn. 3. Bloomfield and Zimm [27] calculated ϕ_c for non-draining linear molecules with various degrees of flexibility, and expressed this as $\phi_c(\varepsilon)$ where ε is related to the a-exponent in the Staudinger-Mark-Houwink equation through $\varepsilon = (2a - 1)/3$. The intrinsic viscosity data of both spectrin dimers and tetramers [11,12,40] yield $a = 1$, $\varepsilon = 0.33$ and $\phi_c = 1.1 \cdot 10^{23}$ mol^{-1}. The value of the excluded volume expansion coefficient α of spectrin dimers at an ionic strength of 10 mM is not known. The light scattering data of Elgsaeter [6] show that the second virial coefficient for spectrin dimers increases sharply when the ionic strength is reduced below 10 mM, but at 10 mM the second virial coefficient is still close to zero indicating that spectrin for this low salt condition may still be close to its unperturbed dimensions for which $\alpha = 1$. This was another important reason for doing the viscosity measurement at this and not a lower ionic strength despite that this would have been desirable in order to make the relative content of spectrin tetramers even smaller. Table I gives the calculated root mean square end-to-end distance for spectrin dimers for some selected temperatures using the Bloomfield-Zimm theory and $\alpha = 1$.

The estimated values of $\langle r^2 \rangle$ obtained from our intrinsic viscosity data (Table I) decrease with increasing temperature. For a polyelectrolytic macromolecule the expansion factor α would be temperature dependent if the Debye shielding distance, λ_D, of the solution was temperature dependent. However, the temperature dependence of the dielectric constant of water [41] is such that λ_D is very close to constant in the temperature range 4–38°C. The observed temperature dependence of the spectrin intrinsic viscosity therefore appears not to be the result of a temperature dependence in α, but in $\langle r^2 \rangle$ as assumed in Table I. When

TABLE I

PERSISTENCE LENGTHS AND END-TO-END DISTANCES OF SPECTRIN

The root mean square end-to-end distance, $\sqrt{\langle r^2 \rangle_2}$, of spectrin dimers calculated according to the Bloomfield-Zimm theory (Eqn. 3), using the experimentally obtained spectrin dimer intrinsic viscosity, $\alpha = 1$ and $\phi_c = 1.1 \cdot 10^{23}$ mol^{-1}. The spectrin dimer persistence length, q_1, is calculated according to the Porod-Kratky model using Yamakawa and Yoshizaki's theory, Eqn. 1 and a spectrin dimer contour length of 100 nm [7]. The spectrin dimer persistence length q_3 is calculated according to Eqn. 2. The root mean square end-to-end distance of spectrin tetramers, $\sqrt{\langle r^2 \rangle_4}$ is calculated using Eqn. 3, the persistence length q_3 obtained for spectrin dimers and a contour length of 200 nm for spectrin tetramers [7].

T (°C)	$[\eta]$ (ml/g)	$\sqrt{\langle r^2 \rangle_2}$ (nm)	q_1 (nm)	q_3 (nm)	$\sqrt{\langle r^2 \rangle_4}$ (nm)
4	43	56.4	25	19.8	84.5
15	38	54.1	22	17.8	80.5
30	37	53.7	21	17.4	79.7
38	34	52.4	20	16.4	77.6

$\langle r^2 \rangle$ for a worm-like macromolecule decreases with increasing temperature, this implies that the internal energy of the molecule decreases with increasing $\langle r^2 \rangle$ [42]. Our viscosity data on spectrin dimer therefore suggest strongly that the increase in free energy associated with making the $\langle r^2 \rangle$ for spectrin longer than the equilibrium value for the free molecule, is of entropic and not energetic origin. The data presented in Table I for spectrin dimers yield $\partial(\ln\langle r^2 \rangle)/\partial T = -(0.004 \pm 0.002)$ K^{-1}, which is close to what is predicted for Porod-Kratky macromolecules [25]. This is another indication that spectrin can be described as a Porod-Kratky chain. It is important to note that the internal energy of such molecules has its minimum when the molecule is straight and fully extended [23]. It is of interest to note that Johnson et al. [43] observed that the diameter of Triton X-100 residues of erythrocyte membranes decreased when the temperature was increased from 0°C to 37°C. This is in semi-quantitative agreement with the observed decreased end-to-end distance of spectrin with increasing temperature. Our viscosity data thus indicate that spectrin is a highly flexible, but not a random coil macromolecule, and that the intramolecular thermal motion of spectrin increases significantly as the temperature is increased. This suggests that the thermal energy is of the same order of magnitude or higher than the energy needed to bend the flexible regions of the spectrin molecules substantially.

The Porod-Kratky model for a worm-like macromolecule (Eqn. 2) can be used to obtain an estimate of the persistence length of spectrin dimers. Such calculations using a dimer contour length of 100 nm and the root mean square end-to-end distance given in Table I yield persistence lengths of 16–20 nm (Table I). The theory of Yamakawa and Yoshizaki [22] can also be used to obtain an estimate of the persistence lengths of worm-like molecules when the intrinsic viscosity and hydrodynamic diameter are known (Eqn. 1). The estimate of the hydrodynamic diameter of spectrin needed to do this calculation was obtained assuming that the molecular weight of spectrin dimer is 460 000 [5], with a density of 1.37 g/ml [44], which yields an estimated diameter of 2.7 nm. The persistence lengths obtained from the intrinsic viscosity data using these two approaches are nearly identical (Table I) and close to the value obtained earlier from electric birefringence relaxation measurements [15]. However, the ionic strength dependence of the intrinsic viscosity of spectrin dimers yields an estimate of the stiffness parameter B introduced by Smidsrød and Haug [45] which indicates an equivalent Kuhn statistical segment length of about 8 nm [11]. This corresponds to a substantially more flexible macromolecule than indicated by the calculation based on the Porod-Kratky model presented here. The reason for this discrepancy is not known.

The root mean square end-to-end distance of spectrin tetramers is estimated to be 78–84 nm assuming a persistence length as calculated for spectrin dimers (Table I). This is in excellent agreement with the mean junction distance of a 6-fold topological replicating network of the erythrocyte membrane skeleton [46,47]. This indicates that the spectrin tetramer end-to-end distance in the erythrocyte membrane may be in the same range as in aqueous solution.

The viscosity data presented here support earlier findings indicating that spectrin is a highly flexible and elongated macromolecule. The additional, new finding that the free energy increase associated with making the end-to-end distance of spectrin

larger than the equilibrium value of the free molecules is of entropic and not energetic origin makes it most likely that the erythrocyte membrane skeleton constitutes an ionic gel where the elastic shear modulus is given by Eqn. 4. There is an excellent agreement between the value of the erythrocyte membrane elastic shear modulus predicted from Eqn. 4 and the experimental value [47]. Waugh and Evans [21] concluded from micropipette aspiration experiments that the erythrocyte membrane shear modulus decreases with increasing temperature. This result together with Eqn. 5 lead to the conclusion that there is a significant reduction in the number of effective network strands in the erythrocyte membrane skeleton when the temperature is increased. This may to some extent be accounted for by a shift in the equilibrium between spectrin dimers and tetramers with temperature (Fig. 2), but some or all of the other non-covalent associations involved in maintaining the membrane skeleton integrity may also be temperature dependent.

The experimental finding that the free energy increase associated with elongation of the spectrin molecules is of entropic and not energetic origin has very important implications for the understanding of the molecular basis for the functional mechanism of spectrin and the erythrocyte membrane skeleton. This suggests that the spectrin molecules carry out important aspects of their functional role simply by being long, thin macromolecules undergoing vigorous intramolecular thermal movements, and that the swollen network of spectrin molecules which makes up the erythrocyte membrane skeleton, therefore most likely constitutes an ionic gel.

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