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Rotational dynamics of erythrocyte spectrin

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The rotational diffusion of erythrocyte spectrin has been measured using time-resolved phsophorescence anisotropy. The anisotropy of the spectrin dimer decays to zero with a time constant of 3 μs at 21°C. The results are compared with the correlation times predicted for the anisotropy decay of an equivalent sphere and rigid rod. The data indicate that the ribbon-like spectrin molecule posesses considerable torsional and segmental flexibility. These motions are restricted, but not abolished, when spectrin is reconstituted into cross-linked cytoskeletal protein networks, or bound to spectrin-actin depleted erythrocyte membrane vesicles.

The submembranous cytoskeletal network of the erythrocyte permits deformation of the membrane without cell lysis during passage through small fenestrations in the microvasculature. It is also responsible for the stability of the cell membrane under conditions of high shear stress such as occur in the heart and major arteries. The cytoskeleton consists of a cross-linked lattice of short actin filaments bound mainly to spectrin and protien band 4.1. Attachment to the membrane inner surface is achieved by spectrin binding to band 2.1 (ankyrin) which is in turn bound to the integral membrane protein band 3 (the anion transporter). In addition, spectrin binds to band 4.1 which itself binds to both glycophorin C and band 3. Ref. 1 provides a recent review of these relationships.

While the physical and chemical properties of individual proteins and their assembly into the membrane skeleton is well documented [1,2], the molecular dynamics and flexibility of both the individual components and the complete structure require further elucidation. It appears likely that spectrin represents a centre of articulation within the cross-linked network thereby allowing flexibility of the complete cytoskeleton. Spectrin is the major constituent of the erythrocyte cytoskeleton and is composed of two polypeptide subunits of molecular weight approx. 225 000 and 240 000 which associate noncovalently and laterally to form an intertwined ribbon-like structure of extended length 100 nm and persistence length 16–20 nm [2–4]. In the intact membrane spectrin exists predominantly as tetramers formed by head-to-head association of heterodimers. The dimeric and tetrameric forms of spectrin have been studied in free solution by a variety of techniques such as light, neutron and X-ray scattering [5,6], electrically induced birefringence relaxation [7,8], viscometry [4,9], circular dichroism [10,11], optical rotatory dispersion and sedimentation velocity [10]. These studies indicate that the spectrin molecule in solution possesses considerable internal flexibility which probably originates at bridging points between relatively rigid α-helical-rich domains which are found as repeat sequences in the primary structure.

The present paper examines the rotational diffusion and internal flexibility of spectrin in solution, in reconstituted cross-linked cytoskeletal networks, and when bound to spectrin-actin depleted membrane vesicles. The aim is to determine the degree to which the internal flexibility is maintained when the molecule is incorporated into the cytoskeletal matrix.

Materials and methods. Erythrocytes were kindly provided by the Red Cross Blood Bank, South Melbourne. Glucose oxidase (type II) was purchased from Sigma Chemical Co. Fluorescein 5-iodoacetamide (FIA), 5-(iodoacetamidoethyl)aminonaphthaline-1-sulfonic acid (IAEDANS), fluorescein 5-isothiocyanate (FITC), and erythrosin 5-iodoacetamide (EIA) were obtained from Molecular Probes Inc.

Abbreviations: EIA, erythrosin 5-iodoacetamide; FIA, fluorescein 5-iodoacetamide; FITC, fluorescein 5-isothiocyanate; IAEDANS, 5-(iodoacetamidoethyl)aminonaphthaline-1-sulfonic acid.

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The dimeric and tetrameric forms of spectrin were prepared according to established procedures [12]. Labelling of purified spectrin with either isothiocyanate or iodoacetamide derivatives of probes caused substantial aggregation of the protein. This was largely prevented by labelling ghost membranes before the selective low ionic strength dissociation of spectrin and actin. For this purpose, ghosts were labelled for 2 h at 4°C with a 12-fold molar excess of probe to spectrin dimer. Unbound probe was removed by centrifugation and washing of the labelled ghosts. Trace amounts of unbound probe were removed from preparations of purified spectrin by dialysis against buffer. Spectrin was purified on either Bio-Gel A15-M or the Superose 6 FPLC system of Pharmacia. Labelled proteins were characterized according to their electrophoretic mobility, labelling ratio and behaviour on gel filtration chromatography. The molar ratio of fluorophore to spectrin dimer was in the range 1.8–2.2. A solution of protein bands 2.1 and 4.1 (1:1, w/w) was prepared by high ionic strength extraction of spectrin-actin depleted erythrocyte membranes [13]. Actin from rabbit muscle was prepared by established procedures [14].

Steady-state fluorescence anisotropy was measured with a Perkin-Elmer LS5 fluorescence spectrometer. Rotational diffusion on the microsecond to millisecond timescale was measured by the technique of time-resolved phosphorescence anisotropy [15] (for review, see Ref. 16). Rotational correlation times were extracted from the anisotropy decays using nonlinear least-squares procedures. Goodness-of-fit was judged from plots of the weighted residuals. Triplicate or quadruplicate measurements were taken for each experimental fluorescence or phosphorescence anisotropy determination.

Results and discussion. The theoretical diffusion coefficients for rotation of a rigid rod of length L and radius r are given by the Broersma equations [17]:

$$D_{1,L} = k_B T/4\pi\eta\, r^2 L \qquad (1)$$

$$D_{2,L} = (3k_B T/\pi\eta L^3)[\ln(L/r) - 1.57 + 7(1/\ln(L/r) - 0.28)^2] \qquad (2)$$

where $D_{1,L}$ and $D_{2,L}$ are the rotational diffusion constants about the long and transverse axes, respectively, η is the solvent viscosity, k_B is Boltzman's constant and T the absolute temperature. The time-resolved anisotropy is given by [18]:

$$r(t) = A_1 \exp(-t/\phi_1) + A_2 \exp(-t/\phi_2) + A_3 \exp(-t/\phi_3) + A_4 \qquad (3)$$

where $\phi_1 = 1/(4D_{1,L} + 2D_{2,L})$, $\phi_2 = 1/(D_{1,L} + 5D_{2,L})$ and $\phi_3 = 1/6D_{2,L}$, and the coefficients A_i depend on the angle between the absorption and emission dipoles of the chromophore and their orientation relative to the axis of rotation. For a rigid rod the dimensions of the

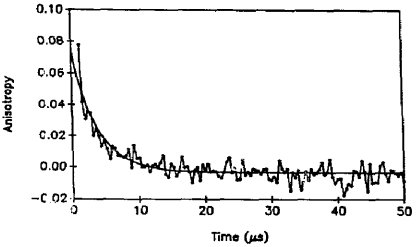

Fig. 1. Time-resolved phosphorescence anisotropy of spectrin dimer labelled with erythrosin-5-iodoacetamide (temp. = 21°C). Excitation was with a nitrogen-pulsed dye laser (8–10 ns pulse width) at 515 nm and the phosphorescence emission was selected with a combination of KV550 and RG695 Schott cutoff filters. The decay was fitted to a single-exponential function: correlation time = 3.7 μs; amplitude = 0.077; apparent zero-time anisotropy (r_0) = 0.074; infinite-time anisotropy (r_∞) = −0.003.

spectrin dimer (100 × 10 nm), the values for these correlation times at 20°C are 1.9, 6.4 and 29.9 μs.

Spectrin dimer in solution (5 mM phosphate buffer, 30 mM NaCl, 0.1 mM EDTA (pH 7.6)) showed a fast decay of phosphorescence anisotropy with a single rotational correlation time of approximately 3 μs and a decay to zero anisotropy within 20 μs (Fig. 1). The experimental correlation times over the temperature range 12–40°C are shown in Fig. 2 and are compared with the correlation times for an equivalent sphere and rigid rod calculated using the Stokes-Einstein equation and Eqns. 1–3, respectively. The experimental correla-

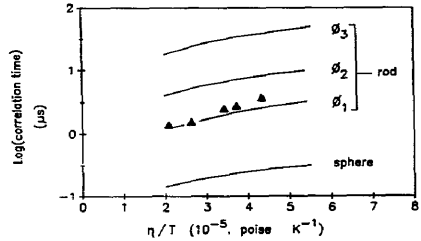

Fig. 2. Rotational correlation times for spectrin dimer as a function of the viscosity/temperature ratio (η/T). The solid lines are the correlation times predicted for an equivalent sphere and for a rigid rod the size of the spectrin dimer (100×10 nm [15,17,20]). The correlation time for the sphere was calculated from the Stokes-Einstein relationship assuming a hydration of 0.37 ml/g. The correlation times for the anisotropy decay of a rigid rod was calculated from Eqn. 3 and the longitudinal and transverse diffusion contansts predicted by the Broersma equations (Eqns. 1 and 2). The symbols refer to the mono-exponential decay found for the spectrin dimer.

tion times are greater by an order of magnitude than those predicted for a spherical protein of the same molecular weight as spectrin. The absence of the two longer correlation times predicted for a rigid rod together with the absence of a finite anisotropy at long times (Fig. 1) suggests that internal motion within the spectrin molecule randomizes probe orientation and depolarizes the emission before phosphorescence is observed. Indeed, the low value of the apparent zero-time anisotropy relative to the limiting anisotropy which characterizes the immobile probe ($r_0 = 0.23$ [19]) is further evidence of such motion. It remains possible, but less probable, that the decay to zero anisotropy and the absence of the two longer correlation times is due to low values of all three coefficients, A_2, A_3 and A_4.

These results are in accordance with published biophysical studies which show that spectrin behaves neither as a rigid rod nor as a compact globular protein [4–11]. The rotational correlation times determined in this study indicate that in addition to axial rotation of the ribbon-like spectrin molecule, faster motions such as intramolecular bending and torsional distortions must contribute significantly to depolarisation of the emitted phosphorescence. Spectrin tetramer behaved in a similar fashion to the dimer with the exception that the anisotropy decays were described by two exponential functions with correlation times of approx. 3 μs and 30 μs. About 80% of this decay arose from the faster component which was similar to that of the dimer, suggesting that a significant part of the measured decay could be accounted for by the internal flexibility of the spectrin dimer.

Measurements of the viscosity [9], the sedimentation coefficient [10] and the light scattering [5] of spectrin indicate that at low ionic strength the molecule undergoes a slight expansion and an increase in its contour length. The root-mean-square end-to-end distance decreases with increasing temperature indicating that the molecule behaves as a typical Porod-Kratky polymer [4]. Circular dichroism measurements show that the expansion at low ionic strength is accompanied by a small loss of α-helical structure in the molecule [10]. The question now arises as to whether these changes at low ionic strength are accompanied by a loss of flexibility.

The rotational correlation time and limiting anisotropy of the spectrin dimer labelled with erythrosin iodoacetamide (5 mM phosphate buffer, 0.1 mM EDTA (pH 7.6)) were found to be independent of NaCl concentration within the range 0 to 100 mM. This lack of ionic strength dependence in the rotational correlation time determined by the phosphorescence technique suggests that internal motions on the phosphorescence time scale remain constant. However, much of the motion responsible for this flexibility may occur on a faster time scale than that of phosphorescence emission. To

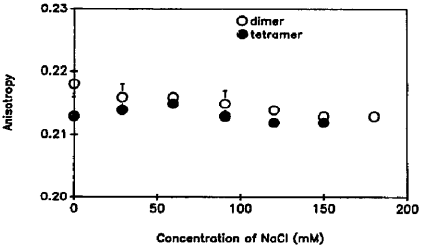

Fig. 3. Steady-state fluorescence anisotropy of spectrin labelled with fluorescein iodoacetamide. Experiments were performed at 10 °C with excitation at 497 nm and emission at 515 nm. Data are presented as mean ± standard deviation. The points without error bars have standard deviations less than the symbol size.

examine this possibility, measurements were made of the steady-state fluorescence anisotropy of dimeric spectrin labelled at thiol groups with fluorescein iodo-

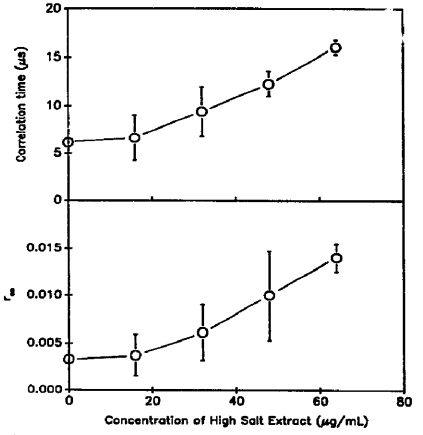

Fig. 4. Rotational correlation time and infinite-time anisotropy (r_∞) of spectrin dimer in the presence of F-actin and the high salt extract consisting of equal amounts (w/w) of bands 2.1 and 4.1. A solution of spectrin (0.6 mg/ml) and G-actin (0.2 mg/ml) was prepared at pH 7.6 (2 mM Tris buffer, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.02% NaN_3). Polymerisation of actin was initiated by the addition of KCl and $MgCl_2$ to 50 mM and 2 mM, respectively. Polymerisation was allowed to proceed at 8 °C for 1.5 h, at which time the solution of bands 2.1 and 4.1 was added. Time-resolved phosphorescence anisotropy experiments were made before and after polymerisation of the actin and during the addition of the band 2.1/4.1 mixture.

acetamide. A small but significant decrease in anisotropy (5 mM Tris (pH 7.6)) occurred on addition of NaCl up to a concentration of 180 mM (Fig. 3). A similar effect was seen for tetrameric spectrin, except that an initial rise in anisotropy was noted up to 30 mM NaCl (Fig. 3). A decrease in anisotropy with increasing salt concentration was also observed for spectrin dimer labelled at amino groups with fluorescein isothiocyanate. In contrast, ionic strength had no effect on spectrin labelled with IAEDANS. This difference cannot be explained by differences in the fluorescence lifetimes of the probes (fluorescein 4 ns, IAEDANS 10–22 ns), and must therefore be due to differences in other characteristics of the probes and perhaps in their wobbling motions at their point of attachment to the protein. It is also possible that different thiol groups were labelled by the different probes due to their differing molecular sizes and ionic states. The fluorescence anisotropy of tryptophan residues in the spectrin dimer increased slightly from 0.085 to 0.092 at 60 mM NaCl thereafter remaining constant. It is clear that the effects of ionic strength on the steady-state fluorescence anisotropy of spectrin are dependent on the type of fluorescence label and on the site of labelling.

The birefringence relaxation time of spectrin dimer and tetramer has been measured by Mikkelsen and Elgsaeter [7,8]. These authors also found that the relaxation rate was much faster than that predicted for a rigid rod, indicating the existence of substantial internal flexibility. At 22°C a single relaxation time of 2 μs was observed for dimer [7], while at 5°C two relaxation times were observed, one of 4 μs, the other of 7–9 μs. These relaxation times were also independent of ionic strength and were similar for the dimer and tetramer species.

Rotational correlation times for spectrin dimer and tetramer were unaffected by the presence of G-actin, or by the polymerisation of the G-actin to its fibrous form. Spectrin is known to bind to F-actin in such a system, and so must remain flexible when attached to this interwoven polymer matrix. Similar findings were made in viscometric studies of spectrin-actin networks [21]. NMR experiments indicated that rapid segmental motions were similar in purified spectrin and in unpurified spectrin-actin extracts from erythrocyte ghosts [22].

When a 1 : 1 (w/w) mixture of bands 2.1 and 4.1 was added to spectrin dimer and F-actin, both the rotational correlation time and limiting anisotropy of spectrin were increased (Fig. 4), but the motion of the spectrin molecule was not frozen and remained faster than that expected on the basis of its molecular size. Band 4.1 potentiates the binding of spectrin to F-actin, and

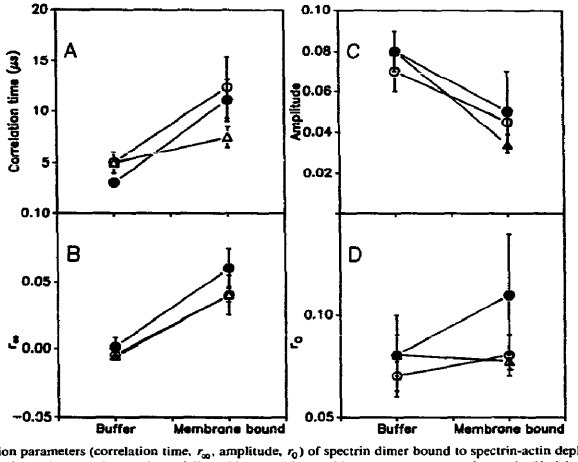

Fig. 5. Rotational diffusion parameters (correlation time, r_∞, amplitude, r_0) of spectrin dimer bound to spectrin-actin depleted membrane vesicles. Vesicles were prepared by low-salt extraction of ghosts followed by extensive washing to remove traces of spectrin. Vesicles and labelled spectrin in 5 mM phosphate, 30 mM NaCl, 0.1 mM EDTA (pH 7.6) were mixed and incubated for 90 min at 21°C. The suspensions were centrifuged in a Fisher microfuge (10 min, 13000 rpm), and the pellets washed and resuspended in the same buffer. ○ and ●, separate experiments at a weight ratio of vesicle protein to spectrin = 1; △, weight ratio = 20.

stabilises the ternary complex [1]. In viscometric studies spectrin was found to remain highly elastic in reconstituted cross-linked systems where it was attached to band 4.1-coated colloidal gold particles [23].

When spectrin dimer was bound to spectrin-actin depleted membrane vesicles (Fig. 5) the rotational correlation time and limiting anisotropy increased, indicating that the motion of spectrin was decreased in rate and restricted in extent. The amplitude of decay decreased, although the decay was not abolished. The increase in the apparent zero-time anisotropy (r_0) indicates a restriction of submicrosecond probe motion which originates from the intramolecular flexibility of the molecule. These data suggest that the uniaxial rotation of spectrin is inhibited on binding to vesicles, while much of the internal flexibility is retained.

Saturation transfer EPR experiments on labelled spectrin also indicate that both dimer and tetramer posess flexibility and local rotational movements [24]. The rotational correlation times of 0.1, 6 and 10 μs were identical for dimer and tetramer and were not abolished by binding to spectrin-actin depleted vesicles [24], however they were slightly reduced if actin also was incorporated [25]. A mathematical model has been developed to account for the interactions of the membrane skeleton and the lipid bilayer in relation to stability and changes in cell shape [26]. Besides the mechanical properties of the lipid bilayer, the major determinant of this model is the flexibility and ionic interactions of the spectrin molecule. The binding of spectrin to vesicles may be mediated by specific attachment to bands 2.1 and 4.1, bound, respectively, to intrinsic membrane proteins band 3 and glycophorin [2]. The spectrin-actin network is sufficiently closely associated with the intact membrane to restrict the lateral diffusion of band 3 [27], allowing further interaction with the membrane bilayer by electrostatic attraction to charged phospholipids [28]. Association of spectrin dimer to tetramer could well be potentiated under these conditions. These events could be expected to dramatically decrease the flexibility of bound spectrin, however, this does not seem to be the case.

In conclusion, the highly flexible spectrin molecule retains significant flexibility in reconstituted cytoskeletal networks and on binding to membrane vesicles. It appears likely that spectrin is the major determinant of the flexibility of the membrane-skeletal structure of the erythrocyte.

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