Transient Dichroism Studies of Spectrin Rotational Diffusion in Solution and Bound to Erythrocyte Membranes†

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ABSTRACT: Spectrin was purified from human erythrocytes and labeled with the triplet probe eosin-5-maleimide. Rotational diffusion of spectrin was investigated by observing transient dichroism following flash excitation of the probe. Measurements were performed at 4 °C in solutions of varying viscosity and with spectrin rebound to spectrin/actin-depleted erythrocyte membranes. In solution, complex anisotropy decays were observed which could not be satisfactorily fitted by the equations for a rod-shaped molecule of appropriate dimensions. When spectrin was rebound to the erythrocyte membrane, a decay in the anisotropy was still present but was markedly less sensitive to solution viscosity and flatter at longer times. In order to overcome the objection that the cytoskeleton is only partially reconstituted when spectrin is rebound, a method was developed for labeling spectrin with eosin-5-maleimide in situ. Anisotropy decays for these labeled membranes exhibited features similar to those obtained for spectrin labeled in solution and subsequently rebound. Taken together, the results provide good evidence for segmental motion of spectrin when incorporated into the erythrocyte cytoskeleton. Upon increasing the temperature, the initial anisotropy r_0 for both rebound and in situ labeled spectrin decreases, and above 30 °C the measured anisotropies are small. Thus, at physiological temperature the probe is almost completely randomized by motions with correlation times less than 10 μs.

The erythrocyte cytoskeleton consists of an assembly of cytoplasmic proteins that impart mechanical strength and control cell shape [for reviews, see Bennett (1985a,b, 1989) and Elgsaeter et al. (1986)]. Spectrin and actin are major components which form a network together with a number of accessory proteins that stabilize the structure and/or attach it to the erythrocyte membrane. Much progress has been made in identifying interactions between the different components by both biochemical and biophysical methods.

Spectrin consists of two subunits, α and β, of M_R = 240K and 225K, respectively. These readily form intertwined heterodimers that can further associate into tetramers by joining together at one specific end through the terminal portions of each subunit (Ungewickell & Gratzer, 1978; Calvert et al., 1980; Tyler et al., 1980; Speicher & Marchesi, 1982). Various experiments indicate that the tetramer is the most likely form on the membrane (Ji et al., 1980; Ralston, 1978; Ungewickell & Gratzer, 1978).

A full understanding of the erythrocyte cytoskeleton requires knowledge not only of how it is assembled but also of its mechanical and dynamic properties. In view of the number of different elements present, this is clearly a complex task. Nevertheless, it is likely that the properties of spectrin are particularly important in determining the overall properties of the network. Electron microscopy of isolated spectrin molecules reveals the heterodimer to be a long rod-shaped molecule about 100 nm in length and 4–6 nm in diameter (Shotton et al., 1979). Since the molecule can adopt a variety of shapes, it is thought to be highly flexible. Both subunits are composed of a large number of homologous domains; the linking regions between domains provide plausible sites of flexibility (Speicher & Marchesi, 1984). Spectrin flexibility is also evident from electrooptic measurements (Mikkelson & Elgsaeter, 1978, 1981). Such flexibility would be functionally important in permitting the cell to deform without rupture in response to the large shear forces experienced in blood capilliaries. An ionic gel model of the erythrocyte cytoskeleton has been proposed to account for the control of cell shape (Elgsaeter et al., 1986).

In the present study, the flexibility of spectrin is investigated by transient dichroism. The technique employs triplet probes to measure rotational diffusion in the microsecond time range. We are able to compare motions occurring within this time window for spectrin (1) free in solutions of different viscosities, (2) reassociated with ghost membranes previously depleted of spectrin and actin, and (3) labeled in situ on ghost membranes. ESR (Cassoly et al., 1980; Lemaigre-Dabreuil et al., 1980; Lemaigre-Dubreil & Cassoly, 1983) and NMR studies (Fung et al., 1986, 1989) have also been reported for spectrin in environments 1 and 2.

Materials and Methods

Preparation of Erythrocyte Ghosts. Erythrocytes were isolated from human blood (O^+) and ghosts prepared by hypotonic lysis according to standard procedures. For preparation of spectrin/actin-depleted ghosts, band 6 was first removed by incubating the ghost volume in 30 volumes of 5 mM sodium phosphate buffer containing 1 mM EDTA, 150 mM NaCl, and 0.2 mM PMSF[1] at pH 7.6 and 0 °C for 30 min. Subsequently, ghosts were incubated in a 0.3 mM sodium phosphate buffer containing 0.2 mM EDTA and 0.2 mM PMSF for 30 min at 37 °C and pH 7.5 as described by Tyler et al. (1980). This releases about 99% of actin and >90% of

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[1] Abbreviations: BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 5PBS, phosphate-buffered saline (5 mM sodium phosphate, 150 mM NaCl, pH 7.5); PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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spectrin, which were removed by centrifugation.

Purification and Labeling of Spectrin. Erythrocytes from 500 mL of fresh blood were twice sedimented in 4 volumes of 5PBS containing 0.75% Dextran 500 at 4 °C. The supernatant and buffy coat were aspirated off. They were then passed down a 3 × 10 cm column comprised of a 1:1 (w/w) mixture of α-cellulose and microcrystalline cellulose (Sigmacell 50) at room temperature under a flow rate of 70 mL/h. This preliminary process ensures the comprehensive removal of white cells and greatly simplifies the task of preventing proteolytic degradation during the subsequent purification.

Spectrin was extracted essentially as described by Ohanian and Gratzer (1984). A total of 50 mL of packed red blood cells was added to an equal volume of 5PBS, containing PMSF (0.05%, w/v). Lysis was induced at 4 °C by the addition of 100 mL of pH 7 buffer containing 0.15 M NaCl, 24 mM HEPES, 1 mM EGTA, 0.5 mM DTT, and 150 mg/mL Triton X-100; 20 mL of this lysate was then layered over 17 mL of pH 7.4 sodium phosphate buffer containing 30% sucrose, 0.6 M KCl, 0.5 mM ATP, 0.5 mM DTT, 24 mM HEPES, and 0.5 mM EGTA, pH 7.4, in a 50-mL centrifuge tube.

The Triton shells were isolated by centrifugation at 80000*g* for 1 h at 4 °C. The pellets were removed, pooled, and washed three times with 10 mM Tris-HCl, pH 7.4, at 25000*g* for 5 min at 4 °C. The volume of the pellet was measured and an equal volume of 2 M Tris-HCl, 0.2 mM DTT, and 0.1% Tween, pH 7.0, added at 25 °C. This suspension was incubated at 30 °C with frequent agitation for 15 min, whereupon it was centrifuged at 100000*g* for 30 min to remove undissolved debris.

The supernatant, which primarily contains band 4.1 and spectrin, was passed down a 120 cm × 2 cm Sepharose 6B gel filtration column equilibrated with 2 M Tris-HCl, 0.1% Tween 80, 1 mM EGTA, and 0.2 mM DTT, pH 7.0, at a flow rate of 40 mL/h. The separation profile monitored at 280 nm followed that described by Ohanian and Gratzer (1984), producing three main protein peaks. The peaks were characterized by SDS–PAGE and respectively found to contain (1) spectrin, (2) spectrin and a trace of 4.1, and (3) 4.1 and some contamination with 4.9.

Fractions collected within peak 1 were pooled, and spectrin was precipitated at 4 °C by the addition of solid ammonium sulfate (31.3 g/100 mL of solution) as described by Bennett (1983). The precipitate was collected after centrifugation at 27000*g* for 20 min and redissolved in 7.5 mM sodium phosphate and 1 mM EDTA, pH 7.0. It was then dialyzed overnight against storage buffer (20% sucrose, 7.5 mM sodium phosphate, 1 mM EDTA, 0.1 mM DTT, and 1 mM sodium azide, pH 7.0) and stored at 4 °C. A total of 50 mL of packed red blood cells typically produced 30–40 mg of spectrin as determined by absorbance measurements.

Spectrin was labeled with eosin-5-maleimide according to the procedure described by Podgorski and Elbaum (1985). The labeling ratio was calculated by first obtaining a Lowry correction factor for unlabeled spectrin from a knowledge of its extinction coefficient, $E_{1\%}^{280} = 10.1$. The correction factor was found to be 2.5 times the BSA value. The correction factor was then applied to the protein concentration as determined by the Lowry method, which, together with the eosin concentration determined by absorbance, yielded the labeling ratio. Eosin-labeled spectrin was dialyzed against the storage buffer and maintained at 4 °C until used.

Reassociation of Eosin-Labeled Spectrin with Spectrin-Depleted Ghosts. Spectrin/actin-depleted leaky ghosts and labeled spectrin were separately dialyzed against rebinding buffer (10% sucrose, 100 mM KCl, 7.5 mM sodium phosphate, 0.1 mM EDTA, and 0.2 mM DTT, pH 7.5). Rebinding was achieved by incubating labeled spectrin (1 mg/mL) with the spectrin-depleted ghosts (2 mg of protein/mL) in rebinding buffer for 3 h at room temperature. Unbound spectrin was removed by layering the ghost suspension over an equal volume of 20% sucrose, 100 mM KCl, and 7.5 mM sodium phosphate, pH 7.5, and then centrifuging at 22000*g* for 20 min. The rebound spectrin membranes were subsequently washed twice with 7.5 mM sodium phosphate, 0.1 mM EDTA, and 0.2 mM DTT, pH 7.4, in preparation for the transient dichroism measurements.

Labeling of Spectrin in Situ. Intact erythrocytes were prelabeled with DIDS essentially as described by Lepke et al. (1976). After labeling, excess DIDS was removed by washing the cells twice in 50 mM Tris-HCl, pH 7.4, containing 110 mM NaCl, 5 mM Na_2SO_4, and 0.5% BSA and then twice more in the same buffer without BSA. Ghosts were then prepared by hypotonic lysis. Spectrin concentration in the ghosts was determined from the total protein concentration, assuming spectrin to comprise 27%. Eosin-5-maleimide was added to ghosts suspended in 5 mM sodium phosphate, pH 7.4 (spectrin concentration 1.14 mg/mL), to a probe concentration of 100 μM and incubated for 1 h at room temperature. Excess probe was removed by repeated washing of the ghosts in the same buffer containing 0.5% BSA followed by two further washes with BSA omitted.

Glutaraldehyde Fixation. A total of 200 μL of ghosts (2–3 mg/mL) was incubated with 2 mL of 1% (v/v) glutaraldehyde in 5 mM sodium phosphate, pH 7.5, for 30 min at room temperature. Glutaraldehyde was removed by four washes with 5 mM sodium phosphate, pH 7.5.

Analytical Ultracentrifugation. Spectrin was suspended in 5 mM sodium phosphate, pH 7.4, at a concentration of 2 mg/mL. The sample was centrifuged for 30–40 min at 4 °C in a Beckman An-D rotor at a speed of 60000 rpm. The protein distribution was analyzed by schlieren optics.

Fluorescence Anisotropy. Fluorescence anisotropy of tryptophan residues of spectrin was measured with a Baird Nova spectrofluorometer. Excitation was at 290 nm, and emission was measured at 335 nm. Fluorescence anisotropy r_f was calculated from

$$r_f = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (1)$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities for emission polarized parallel and perpendicular to the excitation polarization. Spectrin concentrations were typically 8 μM in 5 mM sodium phosphate, pH 7.5, and the intensities were corrected for instrumental factors.

Transient Dichroism Measurements. The rotational mobility of spectrin was measured as previously described (Cherry, 1978; Mühlebach & Cherry, 1985) by observing the transient dichroism of ground-state depletion signals arising from the excitation of the eosin probe by a linearly polarized light pulse from a Nd–Yag laser (JK Lasers, Ltd.). Excitation was at 532 nm by a flash 15–20 ns in duration, and absorbance changes were recorded at 515 nm. Data were analyzed by calculating the absorption anisotropy $r(t)$, given by

$$r(t) = \frac{A_\parallel(t) - A_\perp(t)}{A_\parallel(t) + 2A_\perp(t)} \quad (2)$$

in which $A_\parallel(t)$ and $A_\perp(t)$ are the absorbance changes at time t after the flash for light polarized parallel and perpendicular, respectively, with respect to the polarization of the exciting

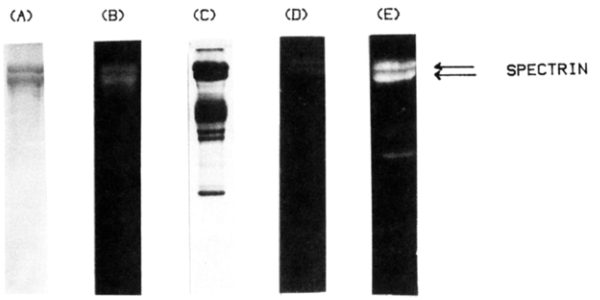

FIGURE 1: SDS–PAGE of eosin-labeled spectrin: (A) purified spectrin—stained with Coomassie Blue; (B) purified spectrin—eosin fluorescence; (C) spectrin/actin-depleted erythrocyte ghosts after rebinding spectrin—stained with Coomassie Blue; (D) as in (C)—eosin fluorescence; (E) erythrocyte ghosts with spectrin labeled in situ—eosin fluorescence.

flash. All results reported here were obtained by averaging 512 signals with a Datalab DL102A signal averager: signal to noise was further improved by combining data from up to four runs on the same sample preparation. Oxygen was displaced from the samples by gently blowing argon over them for 5 min. All transient dichroism measurements were performed in 5 mM sodium phosphate buffer, pH 7.5, with the addition of varying amounts of glycerol. Typical spectrin concentrations were 4–7 μM.

Results

Figure 1A shows SDS–PAGE of purified spectrin and demonstrates that the preparation is essentially free of other proteins. Eosin fluorescence of the gel, shown in Figure 1B, reveals that both the α- and β-subunits are labeled to a similar extent. Preparations with various eosin:spectrin (monomer) mole ratios were obtained in the range 7:1 to 1:1. Ultracentrifugation demonstrated that eosin labeling did not affect the oligomeric state of spectrin when suspended in 5 mM sodium phosphate, pH 7.4, at 4 °C and at a concentration of 2 mg/mL.

Fluorescence of the eosin probe was also useful for monitoring the rebinding of spectrin to spectrin/actin-depleted ghosts, as demonstrated in Figure 1C,D. Labeling of spectrin in situ after blocking the band 3 binding site with DIDS resulted in quite selective location of the eosin probe on spectrin as shown in Figure 1E, although small amounts of label were associated with othere membrane components, particularly band 4.5. The eosin:spectrin (monomer) mole ratio was typically ~2.5:1 for in situ labeled samples.

In order to observe rotation of spectrin in solution on a time scale appropriate to the technique, the viscosity of the medium was increased by addition of glycerol. Some additional experiments were performed in sucrose solutions; the results were similar to those presented for glycerol solutions of equivalent viscosity. Figure 2 shows anisotropy decay curves for eosin-labeled spectrin in solutions of varying viscosity. The shapes of the decay curves are clearly sensitive to the viscosity of the medium. The results in Figure 2 were obtained with the maximum eosin:spectrin ratio of 7:1, which gave the best signal to noise. Qualitatively similar results were obtained with a 3:1 labeling ratio, but the poorer signal quality made it difficult to make a valid quantitative comparison.

Significantly different anisotropy decay curves were obtained with labeled spectrin rebound to the erythrocyte membrane. The curves still exhibit a decay but are flatter at longer times and much less viscosity dependent (Figure 3A). When the anisotropy decay was measured at different temperatures, it was found that both the shape and initial anisotropy, r_0, are

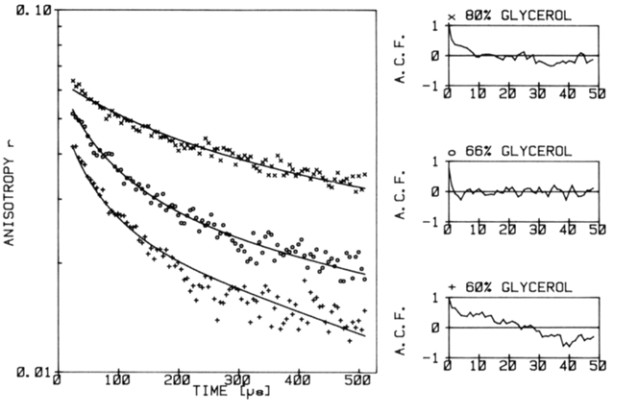

FIGURE 2: Anisotropy decays of eosin-labeled spectrin in glycerol-buffer mixtures at 4 °C: (×) 80% (w/v) glycerol; (O) 66% (w/v) glycerol; (+) 60% (w/v) glycerol. The lines are the best fits obtained from global analysis of the three data sets as explained in the text. The autocorrelation function (ACF) of the residuals for each data set is shown in the panels on the right.

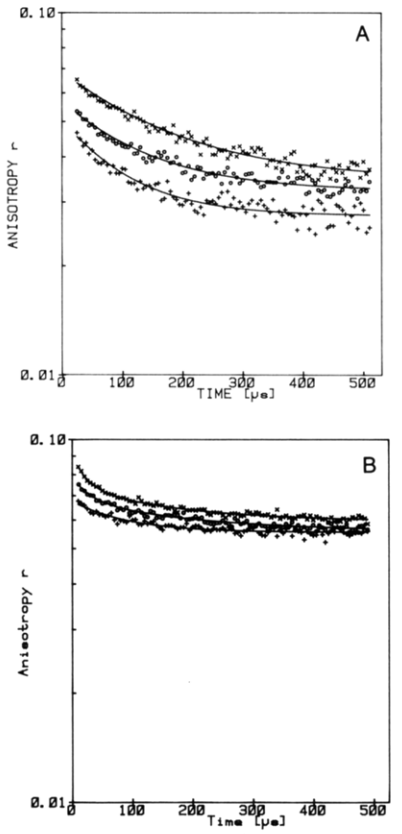

FIGURE 3: (A) Anisotropy decays for eosin-labeled spectrin rebound to spectrin/actin-depleted erythrocyte ghosts and measured in glycerol–buffer mixtures at 4 °C: (×) 80% (w/v) glycerol; (O) 66% (w/v) glycerol; (+) 50% (w/v) glycerol. The lines are least-squares fits by a single exponential plus a constant term. For clarity, the 80% glycerol curve has been displaced upward by 5% and the 66% glycerol curve downward by 5%; otherwise, they are closely coincident. (B) Anisotropy decays for erythrocyte ghosts with spectrin labeled with eosin in situ. Measurements were in glycerol–buffer mixtures at 4 °C: (×) 80% (w/v) glycerol; (O) 66% (w/v) glycerol; (+) 50% (w/v) glycerol. The lines are best fits by either a single or double exponential plus a constant term. For clarity, the 80% curve has been displaced upward by 2% and the 50% glycerol curve displaced downward by 2%.

markedly temperature dependent (Figure 4). The anisotropy in the microsecond time range is very small above 30 °C.

Anisotropy decay curves were also measured for spectrin labeled by the in situ method. The dependence on viscosity at constant temperature is shown in Figure 3B while the temperature dependence is shown in Figure 5. The effect of

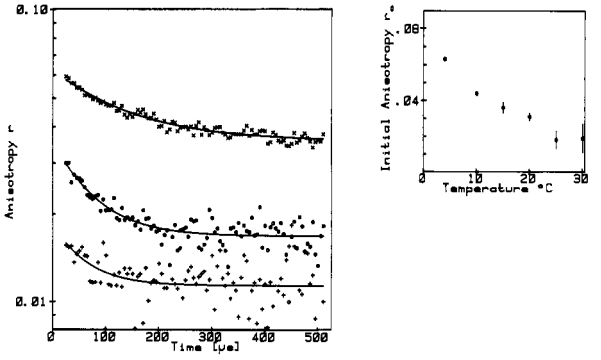

FIGURE 4: Anisotropy decays for eosin-labeled spectrin rebound to spectrin/actin-depleted erythrocyte ghosts and measured in a 66% glycerol–buffer mixture at different temperatures. The left-hand panel shows representative decays measured at (×) 4, (○) 15, and (+) 30 °C. The initial anisotropy r_0 was obtained from the best fits by a single exponential plus a constant term and is plotted against temperature in the right-hand panel. The error bars are ±1 standard deviation obtained from the computer fits.

Table I: Steady-State Tryptophan Fluorescence Anisotropy for Spectrin in Solution

	anisotropy[a]	
temp (°C)	unlabeled	labeled
10	0.145 ± 0.005	0.139 ± 0.005
20	0.122 ± 0.005	0.118 ± 0.005
30	0.102 ± 0.008	0.091 ± 0.006
37	0.070 ± 0.009	0.065 ± 0.007

[a] Eosin:spectrin (monomer) mole ratio = 3:1. Values for both unlabeled and labeled spectrin are the mean and standard deviation of three determinations.

glutaraldehyde fixation on the anisotropy decay of spectrin labeled in situ is also included in Figure 5. After fixation, r_0 is no longer temperature dependent.

The results of steady-state tryptophan fluorescence anisotropy measurements of unlabeled and eosin-labeled spectrin in solution are presented in Table I for a 3:1 labeling ratio. At this ratio, there are no significant differences between the labeled and unlabeled sample over the temperature range 10–37 °C.

Discussion

Spectrin Labeling. In this study eosin:spectrin monomer mole ratios of up to 7:1 were achieved. This is considerably higher than the 2:1 ratio previously reported (Podgorski & Elbaum, 1985) and the 3:1 ratio obtained for a maleimide spin-label (Cassoly et al., 1980) and for ethylmaleimide (Haest et al., 1978). However, since the spectrin monomer contains 15–20 cysteine residues, the attainment of high labeling ratios is not surprising.

Possible perturbation of the spectrin structure by eosin labeling was investigated by measuring steady-state fluorescence anisotropy of tryptophan. No significant difference was found between unlabeled spectrin and eosin-labeled spectrin at 3:1 mole ratio (see Table I). A small increase in fluorescence anisotropy of about 5% was, however, noted at higher labeling ratios (not shown). As a further test, the ability of eosin-labeled spectrin to rebind to spectrin/actin-depleted ghosts was examined. For all labeling ratios, the labeled spectrin rebound satisfactorily and furthermore did not bind to normal ghosts (i.e., not depleted of spectrin/actin). We conclude that a major perturbation of spectrin structure by the labeling procedure is unlikely to have occurred.

Rotational Diffusion of Spectrin in Solution. The dimensions of the spectrin dimer have been determined by electron microscopy to be approximately 100 nm in length and 4–6 nm in diameter (Shotton et al., 1979). The tetramer is formed from a head–head interaction of two dimers and is thus about 200 nm in length.

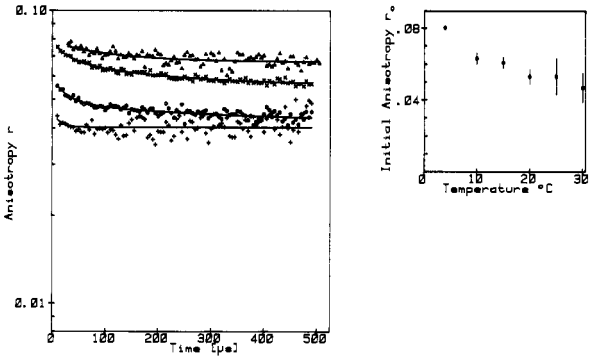

FIGURE 5: Anisotropy decays at different temperatures for erythrocyte ghosts with spectrin labeled with eosin in situ. Ghosts were suspended in a 66% glycerol–buffer mixture. The left-hand panel shows representative decays measured at (×) 4, (○) 15, and (+) 30 °C and at 4 °C after glutaraldehyde fixation (▲). After fixation, the anisotropy decay does not change with increasing temperature. The initial anisotropy for unfixed samples was obtained from best fits by a single exponential plus a constant term and is plotted against temperature in the right-hand panel. The error bars are ±1 standard deviation obtained from the computer fits.

In order to evaluate the anisotropy decays measured in solution, we have modeled spectrin as a cylinder of length $2L$ and radius b freely rotating in a solution of viscosity η. Rotational diffusion coefficients D_1 and D_2 for rotation about the long axis and the transverse axis, respectively, are given by Broersma (1960):

$$D_1 = \frac{kT}{8\pi\eta b^2 L} \tag{3}$$

$$D_2 = \frac{3kT}{8\pi\eta L^3}\left\{\ln(2L/b) - 1.57 + 7\left[\frac{1}{\ln(2L/b)} - 0.28\right]^2\right\} \tag{4}$$

The anisotropy decays are given as the sum of three exponentials:

$$r = A_1 \exp(-t/T_1) + A_2 \exp(-t/T_2) + A_3 \exp(-t/T_3) \tag{5}$$

where

$$T_1 = \frac{1}{4D_1 + 2D_2} \qquad T_2 = \frac{1}{D_1 + 5D_2} \qquad T_3 = \frac{1}{6D_2}$$

The coefficients A_1, A_2, and A_3 are governed by the orientation of the probe transition dipole moment with respect to the molecular axes. In the present case, the relationship is complex because of the existence of multiple labeling sites on spectrin, which probably have nonidentical orientations. However, this does not affect the correlation times, which are determined by the molecular dimensions. Other formulations of the diffusion coefficients can be used but give very similar equations and results.

When this model is fitted by nonlinear least-squares minimization, using cylinder dimensions b and $2L$ and coefficients A_1, A_2, A_3 as variables, the computer-generated errors in the best values are high (e.g., $2L = 190 \pm 200$ nm); the measurements are not sufficiently accurate to resolve three-exponential components even when linked by D_1 and D_2. However, application of global analysis (Knutson et al., 1983) to this problem narrows the errors considerably; in this technique, data sets obtained under different conditions are fitted simultaneously to the same model. Of the variable parameters,

those common to all data sets are termed global, while others apply to just one set and are called individual; in addition, there will be the experimental parameters that are not adjustable.

In this case, the experimental parameter is solution viscosity, adjusted by the percentage of glycerol, while the temperature was held constant. The global parameters were cylinder dimensions b and $2L$ and fractional coefficients $c_2 = A_2/(A_1 + A_2 + A_3)$ and $c_3 = A_3/(A_1 + A_2 + A_3)$, but each data set was given an individual initial anisotropy value $r_0(\alpha)$ (α being the data set identifier) to allow for the variations between measurements due to differences in scattering properties etc. The fittings were performed by a modified nonlinear least-squares procedure on a Digital MINC-11 minicomputer or an Acorn BBC-B+ microcomputer; the fits obtained from such an analysis are illustrated in Figure 2 and yielded values of $2L = 125 \pm 7$ nm and $b = 5.8 \pm 0.8$ nm.

The above value for $2L$ is in reasonable agreement with that from electron microscopy for the spectrin dimer but would be a factor of 2 too small for the tetramer. The value of b is considerably larger than that obtained from electron microscopy (2–3 nm). Spectrin is most probably dimeric under the experimental conditions (Ungewickell & Gratzer, 1978) although the effect of glycerol on spectrin association is not known. Our ultracentrifugation measurements indicate that eosin labeling has no effect on the oligomeric state of spectrin.

It would be rather surprising if analysis of the anisotropy decay did yield accurate dimensions for spectrin since eq 1–3 apply to a rigid cylinder while spectrin is believed to be a flexible molecule. In fact, even the best fits to the rigid cylinder model do not give satisfactory autocorrelation functions (Figure 2), suggesting that the model is inadequate. Equations for the anisotropy decay resulting from torsional motion of a thin flexible rod have been derived by Barkley and Zimm (1979) and used, for example, to analyze anisotropy decays of eosin-labeled F-actin (Yoshimura et al., 1984). In this case, the measured anisotropy decays were much faster than those predicted for a rigid rod so that the contribution from overall rotation could be neglected. This is not the case for spectrin in solution where a full analysis would have to incorporate contributions from both overall and internal flexibility. This would be a complex task, and it is by no means clear that an unambiguous interpretation could be obtained. For present purposes, the solution studies provide a basis for comparison with anisotropy decays measured when spectrin is bound to the erythrocyte membrane.

Rotational Diffusion of Spectrin Rebound to the Erythrocyte Membrane. Transient dichroism measurements were performed with eosin-labeled spectrin rebound to spectrin/actin-depleted ghosts. Samples were suspended in buffer containing glycerol, in part to enable comparisons to be made with the studies of spectrin in solution and in part to obviate contributions from vesicle tumbling, which may arise through the tendency of spectrin/actin-depleted ghosts to fragment.

The anisotropy decays measured with these samples are shown in Figure 3A. It would not be expected that the model of a freely rotating cylinder would apply to rebound spectrin, and indeed, any attempt to fit the data by eqs 3–5 resulted in very poor fits and unrealistic parameters. The decays can in fact be satisfactorily fitted by a single exponential plus a constant term. A constant term is predicted by eqs 3–5 with $D_2 = 0$ and could be interpreted by abolition of end-over-end rotation of spectrin when it is bound to the membrane. Such rotation would be expected to be prevented by multiple attachment to ankyrin and band 4.1 binding sites. However, all global rotation of spectrin should be abolished by these attachments. The fact that a decay in anisotropy is still present is thus good evidence for segmental motion or flexibility of the rebound spectrin. (We checked that there was no dissociation of spectrin from the membrane in the course of the experiment by subsequent centrifugation of the sample.) In this interpretation, the residual anisotropy at long times is likely to be a consequence of the restricted amplitude of segmental motions. However, a contribution from immobile components (e.g., probe molecules located close to spectrin attachment sites) cannot be ruled out. Unlike spectrin in solution, the anisotropy decay for membrane-bound spectrin is rather insensitive to solution viscosity. The anisotropy decays for 80% and 66% glycerol are very similar. (Note that the 80% and 66% glycerol curves in Figure 3 have been displaced in the interest of clarity.) The correlation times for the anisotropy decays in Figure 3 are on the order of 100 μs.

The relatively low values of the initial anisotropy r_0 suggest that motions faster than the time resolution of the experiment are also present. To investigate this effect further, anisotropy decays were measured as a function of temperature. Figure 4 shows that the value of r_0 decreased substantially with increasing temperature. This effect has been observed with other proteins (Bürkli & Cherry, 1981; Junankar & Cherry, 1986) and is most readily interpreted as due to an increasing contribution from fast motions with increasing temperature. For spectrin, the probe is almost completely randomized within 10 μs at temperatures above 30 °C. Cassoly et al. (1980) have also found evidence for temperature-dependent motion in spectrin using spin-labels, while the existence of motions as fast as 10^{-10}–10^{-9} s have been inferred from NMR measurements (Fung et al., 1989).

Rotational Diffusion of Spectrin Labeled in Situ. A possible objection to experiments with rebound spectrin is that the erythrocyte skeleton is not fully reconstituted, and thus, the observed dynamics may differ from the dynamics of the native structure. To overcome this objection, we have developed a method for labeling spectrin with eosin-5-maleimide in situ. This involves first blocking the external eosin binding site on band 3 with DIDS prior to hemolysis. Labeling ghosts from these cells then resulted in quite selective labeling of spectrin as shown in Figure 1E. Selectivity is most likely simply a consequence of the relative abundance of spectrin thiols. We have in fact found that other major erythrocyte components which we have purified, namely, ankyrin, band 4.1, and glyceraldehyde-3-phosphate dehydrogenase, are much less readily labeled by eosin-5-maleimide than is spectrin.

Anisotropy decays measured with spectrin labeled in situ exhibited features in common with those obtained with rebound spectrin (Figure 3B). As with rebound spectrin, the anisotropy decays are markedly less sensitive to solution viscosity and are flatter at longer times when compared with anisotropy decays for spectrin in solution. Fitting the anisotropy decays in Figure 3B to a single exponential plus a constant term again yielded correlation times of the order of 100 μs. A decrease in r_0 with increasing temperature is also seen with in situ labeled spectrin (Figure 5).

There are, however, quantitative differences between the anisotropy decays for rebound specrin and in situ labeled spectrin which require explanation. The anisotropies are higher for in situ labeled spectrin, and r_0 decreases less rapidly with increasing temperature. The fractional coefficient of the decaying component is also smaller. These differences are consistent with spectrin having a more restricted motion in the in situ labeled samples. This suggests that partial reconstitution by rebinding spectrin to spectrin/actin-depleted mem-

branes does not fully reproduce the dynamics of spectrin in the intact cytoskeleton. However, such a conclusion must be treated with some caution. The in situ labeling experiments are not free of criticism since DIDS labeling is thought to have an effect on cytoskeletal properties (Hsu & Morrison, 1983). Indeed, we find that spectrin and actin are resistant to low-salt extraction in DIDS-labeled membranes. In addition to possible perturbation of the cytoskeleton, a small contribution to the anisotropy from non-spectrin-associated probe may occur for the in situ labeled membranes. Thus, the extent to which spectrin motion in the native cytoskeleton differs from that of rebound spectrin remains an open question.

In spite of the quantitative differences between the results obtained from the two methods of labeling, both approaches point to the conclusion that flexibility is an intrinsic property of spectrin when incorporated in the erythrocyte cytoskeleton. Segmental motions are present that can be brought into the microsecond time range at low temperature in glycerol–buffer media. However, at physiological temperature these motions become too fast to measure on a time scale of >10 μs.

The initial anisotropies observed in the present experiments are relatively low even at 4 °C. As discussed in detail elsewhere (Bürkli & Cherry, 1981; Kinosita et al., 1984), independent probe motion is likely to be responsible, at least in part, for these low values. Independent probe motion is, however, much too fast ($\tau_c \approx 1$ ns) to contribute to the anisotropy decays in the time scale used here. To confirm that the present measurements detect motions of the spectrin polypeptide chain, it situ labeled membranes were cross-linked with gluteraldehyde. In these fixed samples, the anisotropy decay at 4 °C was almost abolished, and r_0 was no longer temperature dependent (Figure 5). This result is consistent with the existence of motion of segments of spectrin which are large enough to be cross-linked and hence immobilized by the fixation procedure. However, even after fixation, the anisotropy still has the relatively low value of about 0.08, which indicates that substantial internal motion remains. While this can be explained by independent probe motion, it is conceivable that there are contributions from the motion of segments of the peptide chain containing the probe which are too small to be cross-linked. The NMR observations of Fung et al. (1989) of spectrin motions with correlation times in the order of 10^{-10}–10^{-9} s are in fact indicative of the existence of such highly localized motions.

Flexibility of spectrin is relevant to the rotational diffusion of the integral protein, band 3, in the erythrocyte membrane (Nigg & Cherry, 1979; Austin et al., 1979; Tsuje et al., 1988). Somewhat less than 50% of band 3 molecules have a slow rotational mobility in the millisecond time range, which is thought to be due to restrictions imposed by attachment to the cytoskeleton (Nigg & Cherry, 1980). However, recent studies have cast doubt on this interpretation (Clague et al., 1989). On the basis of the present investigations, it appears that spectrin undergoes rapid molecular motion when attached to the membrane. As a result the cytoskeletal network may be too floppy to provide the necessary restriction to angular displacements of the band 3 proteins over $\pm\pi/2$, which is required to account for the slow component. An attraction of this hypothesis is that it readily explains a long-standing observation that removal of most of spectrin and actin has little or no effect on band 3 rotational mobility (Cherry et al., 1976).

In conclusion, the present transient dichroism measurements are in accord with ESR (Lemaigre-Dubreuil & Cassoly, 1983) and NMR (Fung et al., 1989) studies in revealing the internal flexibility of spectrin. The different techniques have revealed motions in different time domains ranging from about 10^{-10}–10^{-9} s estimated from NMR relaxation times (Fung et al., 1989) to about 10^{-4} s (at 4 °C) in the present study. Taken together, the various measurements suggest that the flexibility of spectrin arises from a complex superposition of segmental motions. Time-resolved fluorescence anisotropy measurements would be of interest both to further characterize the faster motions which are present and to extend the comparison between the results of magnetic resonance and optical spectroscopic studies. While all measurements to date agree that internal motions of spectrin are largely preserved upon binding to the erythrocyte membrane, the present studies are unique in permitting measurement of spectrin labeled in situ, thus avoiding disassembly of the native cytoskeleton. They thus provide the most compelling evidence to date for segmental motion of spectrin while incorporated in the erythrocyte cytoskeleton. Further work in this direction should lead toward a better quantitative understanding of cytoskeletal flexibility and its functional role in controlling the shape and deformability of the erythrocyte.

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Rotational Dynamics of the Ca-ATPase in Sarcoplasmic Reticulum Studied by Time-Resolved Phosphorescence Anisotropy†

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ABSTRACT: We have investigated the microsecond rotational motions of the Ca-ATPase in rabbit skeletal sarcoplasmic reticulum (SR), by measuring the time-resolved phosphorescence anisotropy of erythrosin 5-isothiocyanate (ERITC) covalently and specifically attached to the enzyme. Over a wide range of solvent conditions and temperatures, the phosphorescence anisotropy decay was best fit by a sum of three exponentials plus a constant term. At 4 °C, the rotational correlation times were $\phi_1 = 13 \pm 3$ μs, $\phi_2 = 77 \pm 11$ μs, and $\phi_3 = 314 \pm 23$ μs. Increasing the solution viscosity with glycerol caused very little effect on the correlation times, while decreasing the lipid viscosity with diethyl ether decreased the correlation times substantially, indicating that the decay corresponds to rotation of the protein within the membrane, not to vesicle tumbling. The normalized residual anisotropy (A_∞) is insensitive to viscosity and temperature changes, supporting the model of uniaxial rotation of the protein about the membrane normal. The value of A_∞ (0.20 ± .02) indicates that each of the three decay components can be analyzed as a separate rotational species, with the preexponential factor A_i equal to 1.25× the mole fraction. An empirically accurate measurement of the membrane lipid viscosity was obtained, permitting a theoretical analysis of the correlation times in terms of the sizes of the rotating species. At 4 °C, the dominant correlation time (ϕ_3) is too large for a Ca-ATPase monomer, strongly suggesting that the enzyme is primarily aggregated (oligomeric). From 4 to 20 °C, A_3 decreases markedly (0.43 to 0.15) while A_2 increases (0.14 to 0.40), suggesting a transition to a more mobile (less aggregated) species. At 20 °C, the dominant correlation time (ϕ_2) has a value most consistent with a Ca-ATPase dimer, but a monomer is also possible. Above 20 °C, A_2 decreases in favor of A_1, which has a correlation time (ϕ_1) most consistent with a monomer. Arrhenius plots of protein rotational rates (inverse correlation times) are linear, indicating no abrupt change in protein shape or lipid viscosity. In contrast, the van't Hoff plots of the apparent protein–protein dissociation constants (A_2/A_3 and A_1/A_2) exhibit dramatic slope changes in the range of 18–23 °C, correlating well with changes in the energetics of Ca-ATPase activity and its proposed protein conformational transition. We propose that ATPase monomers and oligomers are in a temperature-dependent equilibrium, with monomers and dimers predominating above 20 °C and higher oligomers predominating at lower temperatures. These results support the hypothesis that protein–protein interaction plays an important role in determining Ca-ATPase activity.

An understanding of the molecular mechanism of active calcium transport in sarcoplasmic reticulum (SR)[1] requires direct information about the molecular dynamics of the Ca-ATPase. One of the fundamental problems that has yet to be resolved is the quaternary structure of the functional ATPase. Protein–protein interactions have been proposed to play an important role in the function of the enzyme. Early observations that only half of the ATPase proteins were capable of forming phosphoenzyme were interpreted as suggesting that

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[1] Abbrevations: ERITC, erythrosin 5-isothiocyanate; DMF, dimethylformamide; DMPC, dimyristoylphosphatidyl choline; 5- and 12-SASL, *N*-oxy-4′,4′-dimethyloxazolidine derivatives of stearic acid; MOPS, 3-(*N*-morpholino)propanesulfinic acid; ST-EPR, saturation transfer electron paramagnetic resonance; SR, sarcoplasmic reticulum.

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