Elasticity of the human red cell membrane skeleton

Effects of temperature and denaturants

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ABSTRACT The molecular basis for the elasticity of the human erythrocyte membrane was explored. Skeletons were released from ghosts in Triton X-100 and their dimensions followed by dark-field microscopy and packed volume. The rest size of skeletons was assumed to reflect the balance point between expansion (deformation) driven by electrostatic repulsions among the excess of fixed negative charges on the proteins and contraction (recovery) driven by their elasticity.

The size of skeletons decreased with increasing temperature. This finding suggests that entropy drives elasticity. The requisite entropy change could be associated with either the configurational freedom of flexible protein chains or with the solvation of side chains exposed during protein dissociation (hydrophobic effects). To distinguish between these two alternatives, we tested the impact of two weak denaturants, 10% ethanol and 20 mM lithium 3,5-diiodosalicylate. Both agents reversibly promoted the expansion of skeletons, presumably by reducing their elasticity. Since the conformation of random coils and globular proteins should not be significantly altered by these mild treatments, this finding strongly suggests a role for weak interdomain and/or interprotein associations.

We conclude that the elasticity of the red cell membrane skeleton may not derive from the configurational entropy of flexible coils. Rather, the elastic energy may arise from reversible dissociations of weak but specific intramolecular and/or intermolecular contacts, presumably within deformed spectrin filaments.

INTRODUCTION

The human erythrocyte is remarkably deformable and very weakly elastic (Evans and Hochmuth, 1978; Hochmuth and Waugh, 1987; Steck, 1989). These properties allow it to maintain both a low viscosity and a stable rest shape as it negotiates the tortuous circulatory system over a period of months (Chien, 1987; Chasis and Shohet, 1987). The membrane is the only solid element in the human red cell and must clearly be the source of its elasticity (Nash et al., 1980). Since lipid bilayers are fluid in plane and do not appreciably resist or recover from shear deformation, the analysis of the elastic properties of red cells has focused increasingly on the skeleton: an irregular network of spectrin, actin, and protein band 4.1 which lines the cytoplasmic surface of the membrane (Marchesi, 1985; Bennett, 1985).

The skeleton, which can be released intact by dissolution of the overlying membrane with mild detergents such as Triton X-100, conserves the rough contour of the cell (Yu et al., 1973). Bearing a net negative charge (Elgsaeter et al., 1976), it expands and shrinks reversibly (elastically) in response to variations in ionic strength (Johnson et al., 1980; Lange et al., 1982). In this study, we seek an understanding of the molecular basis for this elasticity.

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MATERIALS AND METHODS

Ghosts were prepared either in 5 mM NaP_i at pH 7 (Fairbanks et al., 1971) or by lysing cells in 40 vol of 0.02% (wt/vol) saponin in 0.15 M NaCl–5 mM NaP_i at pH 7 (PBS) and washing in PBS. In both cases, $MgSO_4$ was added to the packed white ghosts to make a 0.1 mM solution. The preparations were stored on ice and were used within 3 d. Ghost numbers were determined with a Coulter Counter (model Z_B; Coulter Electronics, Inc., Hialeah, FL) and corrected for coincidence. The pellets contained 6.8×10^9 ghosts/ml (20% SD). Unless indicated, all procedures involving ghosts and skeletons were performed on ice.

Membrane skeletons were prepared by diluting ghost pellets three to tenfold in buffers containing a final concentration of 5 mM NaP_i (pH 7)–0.1 mM $MgSO_4$–4% Triton X-100 (wt/vol) and varied NaCl for 15 min. Skeletons were used only on the day prepared, during which time their properties did not change. Pelleting and resuspending skeletons perturbed them; to avoid this, experiments were performed on unfractionated extracts. Gel electrophoresis was performed as described (Fairbanks et al., 1971; Yu et al., 1973).

Membrane skeleton size was determined in two ways:

(*a*) Dark field microscopy. Skeletons in detergent suspension have been studied by phase-contrast, dark-field, Nomarski and fluorescence microscopy (Liu and Palek, 1980; Johnson et al., 1980; Lange et al., 1982). In the present work, glass slides were precoated by allowing a 50-μl droplet of 1.7 mM poly-L-lysine (M_r 3,800; No. P-0879; Sigma Chemical Co., St. Louis, MO) in 0.1% Triton X-100 to adsorb for 10 min at room temperature. The slide was washed with distilled water and

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air-dried. (When uncoated slides were used, the time required for the settling of skeletons was prolonged, but the results were the same.) Skeletons (10 μl) were applied to the slide in the form of a freshly prepared 10-fold dilution of packed ghosts in Triton X-100 and examined at a total magnification of 1,000 under oil immersion with a Nikon Labophot dark-field microscope. Fixation of samples was achieved by adding glutaraldehyde from a 50% stock to 1% followed by a 30-min incubation at the temperature stated.

Each slide was examined for no more than 30 min during which time no systematic changes occurred. Diameters of photographed skeletons were measured from negatives in a model 6C Shadowgraph micrometer (Nikon, Japan). Usually, at least 50 skeletons were measured for every determination. The standard deviation of repeated measurements of one skeleton was <5%. The standard deviation of populations, shown by the error bars in the figures, was typically ~10%. Skeleton profiles were often slightly elliptical. Therefore, the smallest and largest diameters were measured for each profile and the average of these values presented. The average ratio of the largest to the smallest dimension was 1.12 ± 5% SD.

Statistical analysis was performed using the program RS/1 (BBN Software Products Corp., Cambridge, MA) and an IBM PC-AT computer. Sample populations always showed a normal distribution. Differences between paired samples were taken to be significant at the 95% confidence level.

(*b*) Packed volume measurements were done essentially according to Lange et al. (1982) except that the second (low-speed) spin to level the menicus of the pellets was omitted. Three or more replicate measurements were averaged for every data point. The range of replicate determinations was typically <5%. Assuming unit density and determining the ghost concentration by Coulter count, we calculated a volume (including the trapped extracorpuscular solvent) of 13.8 (±3) μm^3/skeleton for these pellets and 147 μm^3/ghost.

Experimental strategy

The elasticity of the intact red cell (Evans and Hochmuth, 1978; Hochmuth and Waugh, 1987), the isolated ghost (Nash et al., 1980), and gels of purified spectrin (Schanus et al., 1985; Stokke et al., 1986) has previously been explored. The present work examined the meeting point of those studies, the skeleton. Traditional approaches to the measurement of elasticity, such as stress/strain relationships in mechanically stretched specimens (Treloar, 1949; Mark, 1981), were not feasible here. Instead, we measured the size (diameter or volume) of skeletons as a function of the deforming stress imposed by the electrostatic repulsions among the excess of fixed negative charges on the skeleton (Elgsaeter et al., 1976; Johnson et al., 1980; Lange et al., 1982). We reasoned that the rest size of an isolated skeleton would increase until the driving force of coulombic repulsion was balanced by the elastic energy in the network (see Kozlov and Markin, 1987). We varied the electrostatic potential with ionic strength and pH (see Edsall and Wyman, 1958; Tanford, 1961); we varied the elastic modulus of the skeleton with temperature and by the addition of denaturants. We chose conditions where the skeletons were not stretched to the limit, where they were visible and readily photographed in the microscope, where no loss of protein was incurred, and where perturbations were substantially reversible. Our approach did not distinguish among the elasticities of extension, bending and expansion (Evans and Hochmuth, 1978; Hochmuth and Waugh, 1987). Of the two methods used, the microscopic determination of skeleton diameter was the more accurate and direct; packed volume assays risked plastic compression and aggregation of skeletons upon pelleting. Nevertheless, the two methods were in qualitative agreement.

RESULTS

Effects of ionic strength

Fig. 1 shows the response of membrane skeleton diameter and packed volume to the variation of NaCl concentration in the medium. Skeleton size decreased with increasing ionic strength in a fashion reminiscent of classical charge-screening (Tanford, 1961). This premise is also supported by the observation that skeleton diameter decreased 37% when the pH of the buffer was reduced from 8 to 6 (not shown). Microscopy and packed volume determinations gave qualitatively comparable results. The fact that skeleton packed volume paralleled diameter instead of obeying a high-power relationship suggests that the system is complex and is therefore best interpreted only qualitatively (but see Kozlov and Markin, 1987).

The packed volumes of skeletons from ghosts prepared in 5 mM NaP_i agreed well with those of PBS-saponin ghosts which never left the high-salt environment (not shown). This demonstrates that expansion at low ionic strength is reversible, as reported (Johnson et al., 1980; Lange et al., 1982).

It was important in subsequent experiments to be able to preserve skeleton size in the face of experimental manipulations. Fixation with glutaraldehyde was there-

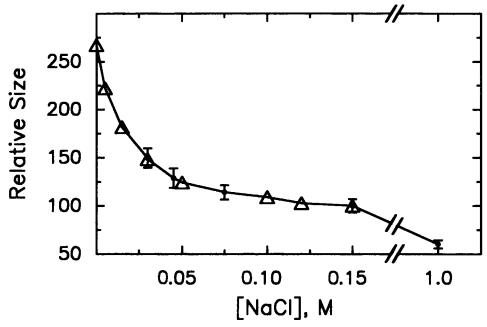

FIGURE 1 Effect of ionic strength on the size of membrane skeletons. For microscopy (↕), ghost pellets were prepared by the PBS-saponin method and diluted twofold into PBS containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). After a 15-min incubation on ice, the suspensions were further diluted with 5 mM NaP_i (pH 7) containing 0.1 mM $MgSO_4$–4% Triton X-100 and NaCl to give the stated concentrations. Samples were incubated for 30 min and fixed on ice, then photographed at ambient temperature. Values were normalized to that at 0.15 M NaCl, which was 2.64 μm in diameter in this experiment. For packed volume measurements (△), ghosts were prepared in 5 mM NaP_i (pH 7) and skeletons liberated in 5 mM NaP_i (pH 7) containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). After incubation for 15 min on ice, the suspensions were brought to the stated NaCl concentrations in the same buffer at a final dilution of threefold. Packed volumes were determined at 4°C as described in Materials and Methods. Values were normalized to that at 0.15 M NaCl, which was 13.8 μm^3 per skeleton.

fore explored as follows: Skeletons in PBS were incubated in glutaraldehyde for 30 min on ice. They were then diluted to low ionic strength and their diameters determined. It was found that glutaraldehyde itself had little or no systematic effect on skeleton size. Fixation prevented a significant change when skeletons were shifted from 150 to 75 mM NaCl. While a shift to 45 mM NaCl provoked expansion, it was only one-third of that experienced by the corresponding unfixed specimen. We conclude that glutaraldehyde fixation is a benign and valuable but not totally effective way to preserve skeleton size.

The dimensions of skeletons were smaller than those of intact cells and ghosts except at very low ionic strength (Lange et al., 1982). This implies that in the intact cell, the skeleton is held open by the membrane bilayer such that the two elements are under balanced tensions at rest (Steck, 1989). A similar but less dramatic situation has been described for peptidoglycan filaments in the cell walls of *E. coli* (Koch et al., 1987).

Effect of temperature

Fig. 2 shows that skeleton volume decreased with increasing temperature at a given ionic strength. Roughly half of the original packed volume was recovered upon return of warmed samples to ice (compare the open and closed symbols in Fig. 2), suggesting that the changes involved both irreversible and reversible components. The reversible component presumably reflected elastic thermocontraction and will be pursued below. The irreversible component might represent plastic remodeling caused by the reformulation of associations among the proteins in the skeleton. Since the skeletons are held in an expanded state in vivo, it is not surprising that they evolve toward a more condensed rest contour when liberated from the bilayer. Control studies revealed that the irrversible change increased with time, temperature, and the reduction of ionic strength. These are the conditions which promote dissociation of spectrin dimer–dimer linkages which could be the rate-limiting step in plastic shape change (Steck, 1989). Experiments were therefore designed to minimize these perturbations as much as possible.

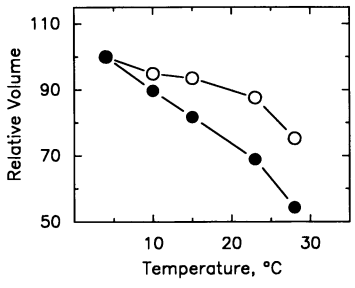

FIGURE 2 Effect of temperature on the size of membrane skeletons: packed volume. Skeletons were prepared from packed PBS-saponin ghosts by dilution with two volumes of PBS containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). After preincubation for 30 min at the stated temperature, the preparations were spun immediately at the same temperature (*solid symbols*) or returned to ice for an additional 30 min before spinning at 4°C (*open symbols*) to test for reversibility.

Fig. 3 shows that the average diameter of skeletons fixed at 22°C (bars *B*) was 13–25% smaller than that of skeletons fixed on ice (bars *A*). Bars *D* show that the thermocontraction was partitially reversible, as observed in Fig. 2. Bars *C* demonstrate that unfixed skeletons were significantly smaller than their fixed counterparts. This extra contraction may be a response to the elevated temperature on the illuminated microscope stage.

Effect of ionic strength on thermocontraction

The diameter of skeletons was determined as a function of NaCl concentration at 0° and 22°C; the degree of thermocontraction is plotted in Fig. 4. The fractional thermocontraction rose hyperbolically with ionic strength. Apparently, the more condensed skeletons were more able to respond to temperature change than their expanded counterparts.

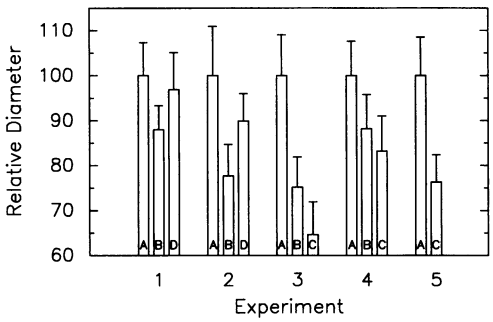

FIGURE 3 Effect of temperature on the size of membrane skeletons: dark-field microscopy. In five independent experiments, skeletons were prepared on ice from PBS-saponin ghosts by a 10-fold dilution in PBS containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). After a 15-min incubation on ice, aliquots were treated in the following four ways (indicated by the labels on the bars): (*A*) Both incubated for 30 min and fixed with 1% glutaraldehyde on ice. (*B*) Both incubated and fixed at 22°C. (*C*) Incubated at 22°C but not fixed. (*D*) First incubated at 22°C and then for 30 min at 0°C; fixed at 0°C. Samples were then photographed at ambient temperature. Values were normalized to condition *A* in each case. The measured diameters (in μm) for *A* were: 2.53, 1.94, 2.21, 2.25, and 2.21; their mean was 2.2 μm ± 11% SD. Differences between the values in each experiment were all significant at 95% confidence except for bars *A* and *D* in experiment 1.

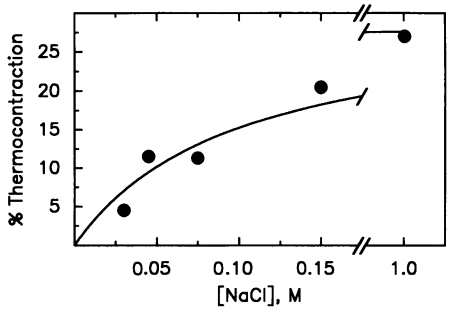

FIGURE 4 Effect of ionic strength on the thermocontraction of the membrane skeletons: dark-field microscopy. Skeletons were prepared by diluting PBS-saponin ghosts in two volumes of PBS–0.1 mM $MgSO_4$–4% Triton X-100 (final). After 15 min on ice, samples were further diluted into 5 mM NaP_i (pH 7) containing 0.1 mM $MgSO_4$–4% Triton X-100 and varied NaCl. Duplicate samples were incubated for 30 min at either 0° or 22°C before fixation at the two temperatures. Samples were photographed and their diameters measured as described in Fig. 1. The values plotted were calculated as 100 (diameter at 0°C minus diameter at 22°C)/(diameter at 0°C). The diameters of the 0°C samples (in μm) were 3.96 ± 0.27, 3.40 ± 0.27, 3.02 ± 0.19, 2.64 ± 0.18, and 1.59 ± 0.11 at the increasing NaCl concentrations shown. A rectangular hyperbola was best-fitted to the data, assuming no change in diameter with temperature at 0 mM NaCl.

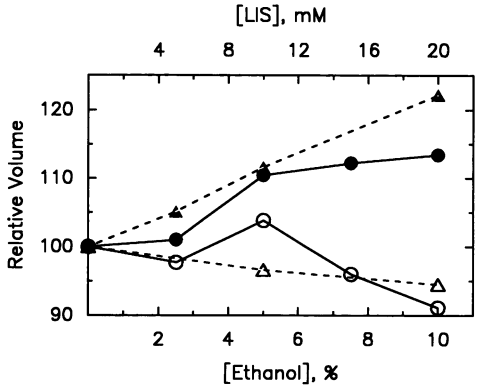

FIGURE 5 Effect of denaturants on the size of membrane skeletons: packed volume. Skeletons were prepared by diluting PBS-saponin ghosts in two volumes of PBS containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). After 15 min on ice, 1 vol of PBS–0.1 mM $MgSO_4$–4% Triton X-100 containing different amounts of ethanol (*circles and solid lines*) or LIS (*triangles and dashed lines*) was added to 2 vol of skeletons. The PBS concentration was preadjusted so that the sum of LIS plus NaCl totalled 0.15 M. Samples were either spun immediately at 0°–5°C and their packed volumes measured (*solid symbols*) or dialyzed overnight in the cold room against PBS containing 0.1 mM $MgSO_4$ before their packed volumes were measured (*open symbols*). Values are plotted relative to the untreated controls.

Effect of denaturants

To test the contribution of protein associations to skeleton elasticity, we sought denaturants which would perturb native structure without significantly altering pH, ionic strength, or dielectric constant, thus keeping constant the electrostatic energy of the skeletons. Furthermore, it was important that the agents not break intermolecular links or otherwise release components of the skeleton. (For example, urea at 0.25 M and guanidine thiocyanate at 0.1 M caused solubilization of a significant fraction of spectrin, as revealed by electrophoresis of the supernatants of centrifuged skeletons.)

Two agents satisfied our criteria: ethanol and the potent chaotrope, lithium 3,5-diiodosalicylate (LIS; see Robinson and Jencks, 1965). As shown in Fig. 5, the packed volume of skeletons increased modestly and reversibly as a function of low concentrations of both of these agents. Since in fact a small decrease in size was observed after subsequent dialysis, the net expansion observed without dialysis probably underestimated the true expansion. Higher concentrations of these agents led to breakdown of the skeletons (not shown).

As with packed volume measurements, the diameter of the skeletons reversibly increased by a small but significant fraction with both 10% ethanol and 20 mM LIS (Fig. 6). Relative expansion increased with temperature; for example, 10% ethanol caused a 17% increase in diameter at 22°C compared to a 7% extension at 0°C.

It could be argued that ethanol caused skeleton expansion not through a direct effect on protein associations but through increasing electrostatic repulsion within the skeleton. This seems unlikely for three reasons: (*a*) While ethanol could increase electrostatic repulsion modestly by lowering the dielectric constant of the medium, offsetting this effect in control experiments by the addition of 0.157 M glycine did not reduce skeleton expansion (see Edsall and Wyman, 1958). (*b*) Ethanol should raise the pK of the charged groups of the proteins, hence decrease anionic charge repulsions and skeleton expansion, (*c*) A quite different agent, LIS, had the same effect as ethanol.

DISCUSSION

We have shown that the size of the isolated red cell skeleton diminishes with temperature and increases in the presence of weak denaturants. We interpret these effects as manifestations of changes in its elasticity and argue that the molecular mechanism responsible is the reversible, hydrophobically driven self-association of spectrin, the principal protein in the network (see Evans and Skalak, 1980, pp. 189–190).

An elastic material stores the energy which deforms it in the reversible reconfiguration of its structure and dissipates that stored energy in the restoration of form once the applied force is removed. Classically, there are

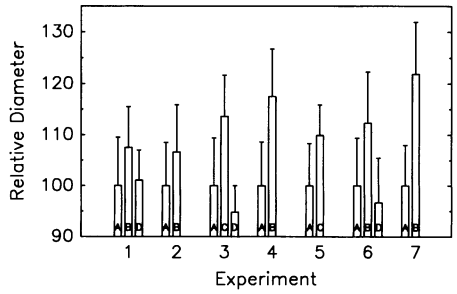

FIGURE 6 Effects of denaturants on skeleton size: dark-field microscopy. In seven separate experiments, skeletons were prepared by diluting PBS-saponin ghosts in four volumes of PBS containing 0.1 mM $MgSO_4$ and 4% Triton X-100 (final). Aliquots were mixed with an equal volume of the same buffer without perturbants (bars *A*), with 10% ethanol (final; bars *B*) or with 20 mm LIS (final; bars *C*). In some experiments (*D*), replicates were then dialyzed overnight at 5°C against buffer to remove the denaturant. In experiments 1–3, samples were incubated for 30 min and fixed at 0°C. In experiments 4 and 5, they were both incubated for 30 min and fixed at 22°C. In experiments 6 and 7, they were incubated for 30 min at 22°C but not fixed. Samples were then photographed at ambient temperature. Values are given relative to those obtained without perturbants, bars (*A*), which had diameters (in μm) of: 2.19, 2.21, 2.35, 1.99, 1.82, 1.88, and 1.67.

two types of elasticity: that of rubber and that of steel (Treloar, 1949; Mark, 1981). Rubbers are networks of crosslinked polymeric chains containing multiple, freely rotating links. The chains become constrained when the network is extended and recover their motional disorder when the stress is released. The elasticity of rubber is thus driven by the gain in configurational entropy of the random coils accompanying release from constraint. In a steel spring, stable associations among some of the atoms are broken upon deformation and reformed upon release; in this case, it is the favorable change in internal energy associated with chemical bonds which drives elasticity. These two fundamental mechanisms are not mutually exclusive; real elastomers such as proteins partake of both (Mark, 1981).

The elastic energy of a network of proteins could derive from their self-association, even though this mechanism works against configurational entropy by constraining the freedom of the chains (Flory, 1956). Such self-association can be driven by favorable changes in both enthalpy and solvent entropy (hydrophobic effects; Tanford, 1980; Privalov, 1979, 1982; Baldwin, 1986). Thus, changes in the entropy of the solvent as well as the configurational entropy of the polymer can drive elasticity. Protein associations have been suggested to underlie the elasticity of the connective tissue protein, elastin (Gosline, 1978; Urry et al., 1986). However, it is the prevailing opinion that protein elastomers are, like rubber, entropic springs (Hoeve and Flory, 1974; Mark 1981).

Spectrin is likely to be the principal elastic element in the red cell membrane, since artificial networks constructed from isolated spectrin are elastic (Schanus et al., 1985; Stokke et al., 1986) and the shear elasticity modulus of the red cell varies in proportion to its spectrin content (Waugh and Agre, 1988). It has been argued that spectrin is an entropic spring (Stokke et al., 1985) and that the skeleton is a gel with elasticity of statistical origin (Elgsaeter et al., 1986). Briefly, the isolated spectrin tetramer has been visualized as a thin and flexible filament ~200-nm long (Shotton et al., 1979; Stokke et al., 1985). Like other flexible polyanions and the skeleton itself, the hydrodynamic radius of purified spectrin varies strongly with ionic strength (Elgsaeter, 1978; Ralston and Dunbar, 1979; Stokke and Elgsaeter, 1981). Since the spectrin molecules do not cross one another in the network but rather join nearest-neighbor actin protofilaments (Byers and Branton, 1985; Shen et al., 1986; Liu et al., 1987), it can be calculated from their numerical density (approximately 1×10^5 tetramers per membrane of 135 μm^2; Steck, 1974) that their average end-to-end length in the intact red cell is ~70 nm, one-third that of their contour length. The consequent redundancy of spectrin chains, combined with their flexibility, could not only foster the high degree of deformability characteristic of the red cell and its membrane but could also confer the configurational freedom needed for entropic elasticity.

Also supporting an entropic mechanism for skeleton elasticity are the observations that both solubilized spectrin (Stokke et al., 1985) and intact skeletons (Figs. 2–3 and Johnson et al., 1980) become more compact with increasing temperature. Thermocontraction is diagnostic of entropy-driven elasticity; rubber contracts with temperature but steel expands (Treloar, 1949; Mark, 1981). Furthermore, the elastic shear modulus of the membrane of the intact cell (Hochmuth and Waugh, 1987) and the ghost (Nash et al., 1980) is very close to that calculated for an ideal entropic network of spectrin filaments (Evans and Skalak, 1980; Elgsaeter et al., 1986).

For the measured elasticity of the skeleton to derive entirely from changes in configurational entropy, however, the filaments must have ideal flexibility (Treloar, 1949; Flory, 1956; Mark, 1981). In rubber, the flexible links are quite unhindered and typically number in the hundreds per polyisoprene chain. In comparison, spectrin is highly constrained. Both side chain steric hindrance and secondary structure limit the motion of a polypeptide backbone; in the case of spectrin, the alpha-helix content is ~70% (Schechter et al., 1976; Calvert et al., 1980). Spectrin also appears to have a folded substructure. Its contour length is approximately one-third that of an alpha-helix of the same mass. Both the spectrin polypeptides appear to be organized into ~18 homologous and independently folded domains, each of 106 residues (M_r

12,000); these are thought to be connected by short disordered regions, like beads on a string (Speicher, 1986). Furthermore, the two chains of the spectrin dimer associate specifically and wrap around each other (Shotton et al., 1979; Marchesi, 1985), conferring additional stiffness (Flory, 1956). In contrast, another filamentous actin-binding protein, caldesmon, appears far more than spectrin to be a random coil: caldesmon is thin and flexible, lacks significant secondary structure, and has a length/mass twice that of spectrin (Lynch et al., 1987; Graceffa et al., 1988).

There is also much physical evidence documenting a folded structure for spectrin (Schechter et al., 1976; Brandts et al., 1977; Calvert et al., 1980; Yoshino and Marchesi, 1984; Minetti et al., 1986). Magnetic resonance in particular has suggested that most of the spectrin side chains are immobilized in a readily reversible state (Cassoly et al., 1980; Lammel and Maier, 1980; Fung et al., 1986). Finally, electron microscopy has revealed that whereas spectrin in intact skeletons can be expanded at low ionic strength to nearly its full contour length, the least perturbed skeletons are condensed; the spectrin is compact rather than elongated (Shen et al., 1986).

If the structure of spectrin prohibits the motional freedom needed to provide membrane elasticity through changes in configurational entropy, another source of energy must be postulated. This could derive from reversible associations of spectrin. In that case, the molecular organization which limits the configurational entropy of the spectrin polypeptide would also provide its elastic energy (Shen et al., 1986). The extension of spectrin in response to mechanical stress need not cause its denaturation but merely the reversible dissociation of independently folded, weakly associated domains either within spectrin molecules or between neighbors. Such intramolecular unfolding reactions are well known in simple hinged proteins (Takahashi et al., 1981; Privalov, 1982; Janin and Wodak, 1983; Horowitz and Criscimagna, 1986).

According to this view, the observed thermocontraction could be driven by the entropy of the solvent rather than the polypeptide: the hydrophobic effect. To test this hypothesis, we examined the effect of denaturants, since they should not affect the configurational entropy of random chains (Brandt and Flory, 1965) but should promote the exposure of buried apolar side chains to water. By fostering spectrin dissociations, the denaturants should reversibly reduce skeleton elasticity, thereby promoting expansion. This was our observation (Figs. 5 and 6). Also consistent with this hypothesis is the presence of multiple hydrophobic sites on the surface of native spectrin, the number of which increases with the expansion of the molecule (see Isenberg et al., 1981).

The concentration of ethanol needed for expansion of the skeletons was much lower than that required to denature most globular proteins (Brandts and Hunt, 1967; Tanford, 1968). This effect suggests that the ethanol promoted the separation rather than the denaturation of weakly associated, independently folded domains within spectrin. Previous work has also indicated that purified spectrin is unusual in undergoing changes in conformation in response to urea or guanidine · HCl at low concentrations (Schechter et al., 1976; Calvert et al., 1980; Yoshino and Marchesi, 1984) as well as other minor perturbations (Cassoly et al., 1980; Lammel and Maier, 1980; Fung et al., 1986). Consistent with this weak self-association is the extreme compliance of the red cell membrane: from a shear elasticity of 0.007 erg/cm^2, it can be calculated that a 100% extension of the skeleton is accompanied by an increase in free energy of only 1.6 Kcal/mol of spectrin tetramers (Hochmuth and Waugh, 1987). Appropriately, this value is an order of magnitude smaller than the free energy of denaturation of globular proteins of modest size (Privalov, 1979, 1982).

Enthalpy changes upon deformation also would suggest protein associations but not configurational entropy as the mechanism underlying skeleton elasticity. Direct evidence is wanting. However, isolated spectrin is known to undergo minor endothermic transitions in the 0–37°C range; these could be associated with the reversal of interdomain contacts (Ralston and Dunbar, 1979; Minetti et al., 1986). As is generally the case in protein unfolding reactions, the small free energy change for spectrin extension could signify the balance between large changes in enthalpy, configurational entropy, and solvent entropy (Privalov, 1979, 1982; Schellman, 1987).

A discrepancy exists between the thermocontraction observed in both isolated spectrin (Stokke et al., 1985) and isolated skeletons (see Results and Johnson et al., 1980) and the thermoexpansion seen in micropipet aspiration studies on intact cells (Waugh and Evans, 1979). One explanation could derive form the temperature-dependence of the dissociation of spectrin tetramers to dimers which would soften the skeleton without reducing the stiffness of the spectrin itself (Kozlov and Markin, 1987). Alternatively, the discrepancy could reflect the variation of the elasticity of spectrin with extension, as commonly observed with other elastomers (Mark, 1981). We see in Fig. 4 that thermocontraction became negligible when skeletons were expanded at low ionic strength. Similary, skeletons were 2.6-fold more dilatable by 10% ethanol when condensed at pH 6 than when expanded at pH 8 (data not shown). In the intact cell, the skeleton affixed to the bilayer is at least as expanded as observed at [NaCl] = 0 in Fig. 4. This degree of extension could prevent the skeleton from underoing thermocontraction in situ. Instead, the enthalpy component of the elastic free

energy of the extended skeleton in the intact cell would favor thermoexpansion, as observed by Waugh and Evans (1979).

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