

Erythrocyte Membrane Deformability and Stability: Two Distinct Membrane Properties That Are Independently Regulated by Skeletal Protein Associations

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Abstract. Skeletal proteins play an important role in determining erythrocyte membrane biophysical properties. To study whether membrane deformability and stability are regulated by the same or different skeletal protein interactions, we measured these two properties, by means of ektacytometry, in biochemically perturbed normal membranes and in membranes from individuals with known erythrocyte abnormalities. Treatment with 2,3-diphosphoglycerate resulted in membranes with decreased deformability and decreased stability, whereas treatment with diamide produced decreased deformability but increased stability. N-ethylmaleimide induced time-dependent changes in membrane stability. Over the first minute, the stability increased; but with continued incubation, the membranes became less stable than control. Meanwhile, the deformability of these membranes decreased with no time dependence. Biophysical measurements were also carried out on

pathologic erythrocytes. Membranes from an individual with hereditary spherocytosis and a defined abnormality in spectrin-protein 4.1 association showed decreased stability but normal deformability. In a family with hereditary elliptocytosis and an abnormality in spectrin self-association, the membranes had decreased deformability and stability. Finally, membranes from several individuals with Malaysian ovalocytosis had decreased deformability but increased stability.

Our data from both pathologic membranes and biochemically perturbed membranes show that deformability and stability change with no fixed relationship to one another. These findings imply that different skeletal protein interactions regulate membrane deformability and stability. In light of these data, we propose a model of the role of skeletal protein interactions in deformability and stability.

THE red cell, as it continuously circulates, must be able to undergo extensive passive deformation and to resist fragmentation. These two essential qualities require a highly deformable yet remarkably stable membrane. The property of membrane deformability determines the extent of membrane deformation that can be induced by a defined level of applied force. The more deformable the membrane, the less applied force necessary to allow the cell to pass through capillaries much smaller than the cellular dimensions. Membrane stability, on the other hand, is defined as the maximum extent of deformation that a membrane can undergo beyond which it cannot recover completely its initial shape, i.e., the point at which it fails. Normal membrane stability allows erythrocytes to circulate without fragmenting, while decreased stability can lead to cell fragmentation under normal circulatory stresses.

The red cell membrane has been well characterized biochemically and is composed of a lipid bilayer, integral proteins, and a skeletal protein network of spectrin, actin, ankyrin, tropomyosin, and proteins 4.1 and 4.9. The membrane skeleton underlies the bilayer and is associated with it by pro-

tein-protein and protein-lipid interactions (3, 4, 7, 18). Although much is known about the biochemical associations in the membrane, the manner in which these associations influence membrane biophysical properties is less well defined. The skeletal protein network is felt to play an important role in determining membrane deformability and stability (8, 10, 16, 22, 28). Evidence to support this contention has been derived from membrane stability studies of red cells from selected patients with defined quantitative or qualitative skeletal protein abnormalities (17, 19, 21, 29, 32, 34). Whether membrane deformability is also perturbed in red cells with these abnormalities is not yet clarified. An important unanswered question is whether membrane deformability and stability are regulated by the same or different skeletal protein interactions. To address this question, we have pursued two lines of investigation. First, we have biochemically perturbed skeletal protein interactions in normal cells and measured the effect of this perturbation on membrane deformability and stability. Second, we have measured these two membrane physical properties in red cells from individuals with known erythrocyte abnormalities.

In both biochemically perturbed normal membranes and pathologic membranes, we found that decreased deformability can be associated with either increased or decreased membrane stability. In addition, normal deformability can occur with decreased stability. These results imply that different skeletal protein interactions regulate the two essential membrane properties of deformability and stability. We will consider these results in light of a model based on known skeletal protein associations and identify the key skeletal protein associations that regulate membrane deformability and stability.

Materials and Methods

Reagents

Alpha chymotrypsin was purchased from Worthington Biomedical Corp., Freehold, NJ; trypsin from CooperBiomedical Inc., Malvern, PA; 2,3-diphosphoglycerate (2,3-DPG)¹, N-ethylmaleimide (NEM), and diamide from Sigma Chemical Co., St. Louis, MO; dextran from Pharmacia Fine Chemicals, Uppsala, Sweden; Stractan from St. Regis Paper Co., Tacoma, WA; Affi-Gel 15 and Affi-Gel 10 from Bio-Rad Laboratories, Richmond, CA.

Preparation of Polyclonal Antibodies

Proteins 4.1 and spectrin were purified by the methods of Tyler (30) and Morrow (24), respectively. The purified proteins were then injected subcutaneously into rabbits in the presence of complete Freund's adjuvant. 4 wk later, the rabbits were boosted intramuscularly and then the sera obtained at 2 wk. Monospecific IgG was prepared from the sera by passage over Affi-Gel 10 coupled to protein 4.1 and Affi-Gel 15 coupled to spectrin. The specificity of the purified IgG was evaluated by immunoblot analysis as described later.

Preparation of Resealed Erythrocyte Membranes

Resealed membranes for deformability and stability measurements were prepared by a procedure adopted from Johnson (13). Blood from normal volunteers and patients was drawn into heparinized or acid citrate dextrose tubes and the erythrocytes collected and washed three times in 5 mM Tris and 140 mM NaCl (pH 7.4). The erythrocytes were then lysed in 40 vol of 7 mM NaCl and 5 mM Tris (pH 7.4). The membranes were pelleted by centrifugation, resuspended in 10 vol of 5 mM Tris and 140 mM NaCl (pH 7.4), and incubated 30 min at 37°C for resealing. Finally, the resealed membranes were pelleted by centrifugation and prepared for ektactometry as described below.

Resealed membranes from normal red cells were biochemically perturbed by incubating with either 0.1–5.0 mM NEM or diamide for varying periods of time at 37° or 4°C. The treated membranes were then assayed in the ektactometer.

Entrapment of Skeletal Perturbing Agents within Resealed Membranes

If the membranes were to be resealed in the presence of a perturbing agent, then the agents listed below were included in the resealing buffer and the lysed membranes were equilibrated in this buffer for 10 min at 4°C and then resealed for 1 h at 37°C. The agents and their concentrations were 1–25 mM 2,3-DPG, and 1–50 ng/ml trypsin.

The reversibility of the 2,3-DPG effect was studied by relaying an aliquot of 2,3-DPG-treated membranes and then resealing them in 5 mM Tris, 140 mM NaCl (pH 7.4) with and without 2,3-DPG, and examining them by ektactometry.

Membrane Deformability Measurements

Resealed membranes, prepared as described above, were suspended in 3 ml of Stractan II (290 mosmol, 22 centipoise [pH 7.4]; St. Regis Paper Co.)

1. *Abbreviations used in this paper:* 2,3-DPG, 2,3-diphosphoglycerate; DI, deformability index; H.E., hereditary elliptocytosis; NEM, N-ethylmaleimide.

and examined by ektactometry, a laser diffraction method previously described (20). In brief, suspended cells are exposed to an increasing shear stress (0–125 dynes/cm²) and the change in their laser diffraction pattern from circle to ellipse measured. This photometric measurement produces a signal designated deformability index (DI) which quantitates cell ellipticity. By an automatic image analysis system, the DI is recorded as a continuous function of applied shear stress. For resealed membranes, the shear stress required to obtain a defined value of DI is determined by the property of membrane deformability without contributions from either internal viscosity or cell geometry (12). There is a correlation between changes in deformability measured by this technique and those measured using the micropipette (6, 9). Analysis of the DI curve generated by the ektactometer thus provides a measure of membrane deformability (21).

Membrane Stability Measurements

Resealed membranes were pelleted by centrifugation and 100 μ l of 40% membrane suspension was then mixed with 3 ml Dextran (40,000 mol wt, 35 g/100 ml in 10 mM phosphate buffer, viscosity 95 centipoise [pH 7.4]), and subjected to a continuous applied shear stress of 575 dynes/cm² in the ektactometer (21). Under this stress, the membranes progressively fragment, generating undeformable spherical fragments. This process is detected as a time-dependent decrease in DI. The time required for the DI to fall to 60% of its maximum value is termed T₆₀ and is taken as a measure of membrane stability.

To evaluate the protein composition of the red cell fragments generated by shear stress, the cell suspension sample was removed from the ektactometer and a population enriched for the smallest fragments was obtained by gravity sedimentation. When the supernatant and the sedimented fractions were examined microscopically, the supernatant contained the small fragments and the residual, large membrane fragments were in the sedimented fraction. The protein contents of the supernatant sample, the sedimented sample, and unsheared whole membranes were then compared using SDS PAGE as described below.

SDS PAGE

Samples for electrophoresis were solubilized in 0.5 M Tris-Cl (pH 6.8), 1.25% SDS, and 0.38 M dithiothreitol, heated to 100°C for 3 min, and analyzed in the discontinuous system described by Laemmli (14) on slab gels composed of a separating gel of 10% acrylamide and a stacking gel of 3% acrylamide. After electrophoresis, the gels were fixed and stained for protein with Coomassie Blue.

Quantitation of Membrane Proteins and Lipids

To determine the relative concentrations of the various proteins in the intact membranes and the shear-generated fragments, a pyridine dye elution method was used (1). To determine the relative concentrations of lipids, the lipids were extracted using the method of Rose and Oklander (25). Then cholesterol was measured by the method of Zlatkis et al. (35), and lipid phosphorus by the method of Bartlett et al. (2).

Immunoblot

Membrane proteins were electrophoretically transferred from polyacrylamide SDS gels to nitrocellulose membranes as previously described (5). Unbound reactive sites were blocked by incubation in 3% BSA in 20 mM Tris and 500 mM NaCl (pH 7.5) for 30 min at 40°C. The membrane was then incubated in specific antibody (15–50 μ g/ml) in 3% BSA, 20 mM Tris, and 500 mM NaCl (pH 7.5) for 2 h at room temperature, washed twice with 20 mM Tris and 500 mM NaCl, and twice with 20 mM Tris, 500 mM NaCl, 0.05% Nonidet P-40, and then incubated in ¹²⁵I-protein A (2.5 \times 10⁵ cpm/ml) in 3% BSA, 20 mM Tris and 500 mM NaCl (pH 7.5) for 1 h at room temperature. Finally, the nitrocellulose was washed as above, dried, and exposed to x-ray film.

Results

Analysis of Shear-induced Fragments

To show that the stability assay used in these studies indeed measures shear-induced disruption of skeletal protein interactions, we first generated fragments by exposing normal, resealed membranes to the constant high shear stress. We

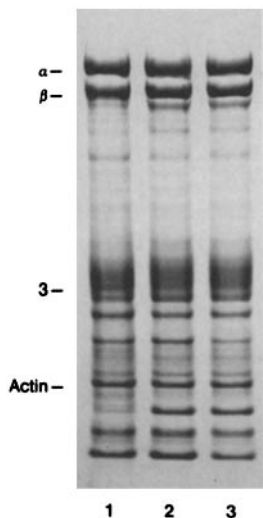


Figure 1. SDS PAGE analysis of membrane fragments and residual membranes generated during membrane stability assay. Lane 1, Coomassie Blue pattern of the small shear-induced fragments collected in the supernatant fraction of 1 g sedimentation. Lane 2, the residual large membrane fragments collected after shearing. Lane 3, the unsheared control membranes. It can be seen that all the major skeletal protein components are present in the three samples. When the membrane proteins were quantitated, the ratios of spectrin, protein 4.1, and actin to band 3 in the fragments, residual membranes, and intact membranes were within 7% of each other.

then examined the skeletal protein content of these fragments by SDS PAGE. Fig. 1 shows that the membrane protein composition of these fragments was the same as that of both un-sheared resealed membranes and of residual membranes after fragmentation. When the membrane proteins of the fragments, residual membranes, and intact membranes were quantitated, the ratios of spectrin, protein 4.1, and actin to band 3 were within 7% of each other. In addition, the protein-to-lipid ratios were also very similar for these three preparations. This indicates that shear-induced fragmentation results in the disruption of protein-protein interactions generating spherical vesicles with the same protein composition as that of the intact membranes from which they were derived.

Effect of Polyphosphates

To evaluate the effect on membrane stability of disruption of skeletal protein associations we entrapped 2,3-DPG within resealed membranes. This polyphosphate is known to dissociate spectrin-actin and spectrin-protein 4.1 interactions (26, 33). The membrane stability of membranes resealed in the presence of 2,3-DPG, in increasing concentrations, is shown in Fig. 2 *b*. In this figure, the DI, at constant applied shear stress, is plotted as a function of time. The DI is a measure of membrane ellipticity, and it decreases as a function of time as the membrane fragments into nondeformable spheres. The fragmentation pattern of normal membranes is shown in the top curve. Treatment of membranes with increasing concentrations of 2,3-DPG caused a dose-dependent increase in fragmentation rate, in other words, a decrease in membrane stability. The membranes treated with 2 mM 2,3-DPG had 0.91 times the normal stability while the 5, 10, and 15 mM treated membranes had 0.71, 0.36, and 0.17 times the normal stability.

To determine whether these changes in stability were reversible, membranes were first resealed in 7.5 mM 2,3-DPG, then lysed and resealed in either 0 or 7.5 mM 2,3-DPG. As shown in Fig. 2 *c*, curve B, the cells that were resealed first in 2,3-DPG, lysed, and then resealed without the polyphosphate, reverted back towards normal stability. In contrast, the cells that were exposed to 2,3-DPG during both resealing steps or only in the second resealing step showed a decreased stability.

Resealing membranes in increasing concentrations of 2,3-DPG also resulted in a dose-dependent decrease in membrane deformability as measured by ektacytometry. The change in DI with increasing concentrations of 2,3-DPG is shown in Fig. 2 *a*. In this figure, the DI is plotted on a linear

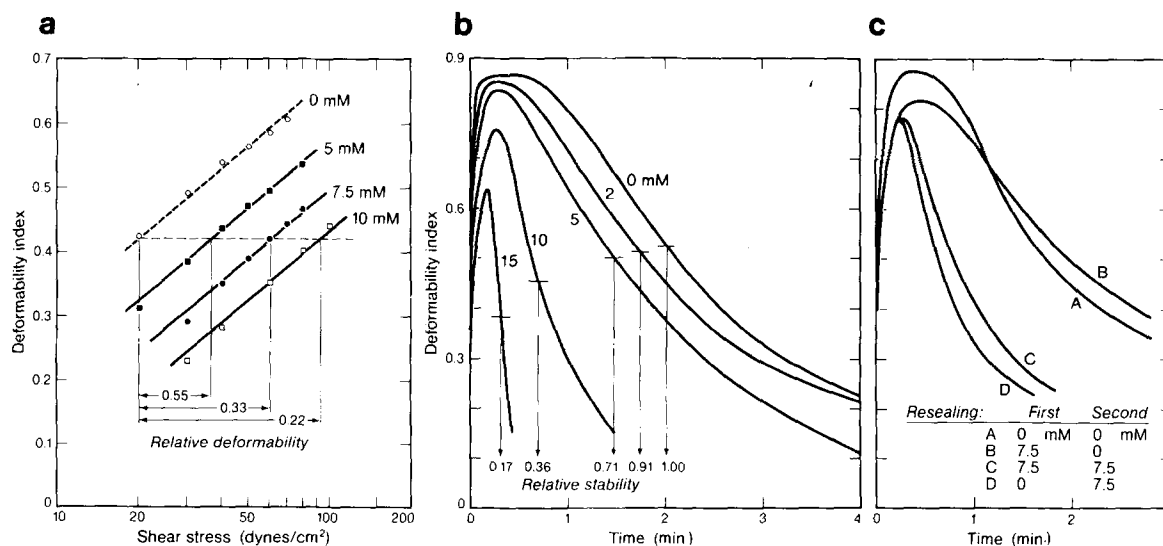


Figure 2. Effect of 2,3-DPG on membrane deformability and stability. (a) The deformability data with the DI plotted on a linear scale and the shear stress plotted on a log scale. Because the lines are parallel, one can see that membranes treated with 5 mM 2,3-DPG required 1.8-fold greater shear stress than normal membranes to reach equivalent deformation at all points along the curve, indicating that the 2,3-DPG-treated membranes had 0.55 times the normal deformability. Treatment with 7.5 and 10 mM 2,3-DPG resulted in membranes that had, respectively, 0.33 and 0.22 times the normal deformability. (b) The stability data with the DI plotted as a continuous function of time. The time required for the DI to fall to 60% of its maximum value is termed T_{60} and is used to compute relative membrane stability. The membranes treated with 2 mM 2,3-DPG had 0.91 times the normal stability and the 5, 10, and 15 mM had 0.71, 0.36, and 0.17 times the normal stability. (c) The reversibility of the 2,3-DPG effect on membrane stability. The decreased membrane stability caused by 2,3-DPG (curve C) was shown to be reversible by lysing the membranes and resealing them in buffer without 2,3-DPG (curve B).

scale and the shear stress plotted on a log scale. The deformability of normal membranes is shown in the top curve. Treatment of membranes with increasing concentrations of 2,3-DPG caused a dose-dependent decrease in the DI obtained at all values of applied shear stress. Since the lines are parallel, one can calculate that membranes treated with 5 mM 2,3-DPG required 1.8-fold greater shear stress than normal membranes to reach equivalent deformation at all points along the curve, indicating that the 2,3-DPG-treated membranes had 0.55 times the normal deformability. Treatment with 7.5 and 10 mM polyphosphate resulted in membranes that had, respectively, 0.33 and 0.22 times the normal deformability.

Effect of Proteolytic Enzymes

To determine whether intact skeletal proteins were required for normal membrane stability, we entrapped increasing concentrations of trypsin within resealed membranes and measured their stability by ektacytometry. As Fig. 3 shows, trypsin, in concentrations from 5–50 ng/ml, caused a dose-dependent decrease in stability. The membranes treated with 5 ng/ml had 0.77 times the normal stability, while those treated with 10 and 25 ng/ml had 0.67 and 0.42 times the normal stability.

To examine the effect of these low concentrations of trypsin on membrane proteins, we ran SDS polyacrylamide gels of membranes resealed in the presence of this enzyme and then analyzed them by Coomassie Blue stain and immunoblot. As can be seen in Fig. 4, with increasing concentrations of trypsin, several new bands appeared with molecular weights less than that of β -spectrin. An immunoblot prepared with anti-spectrin IgG (Fig. 5 A) confirmed that several bands had been generated by digestion of spectrin. The Coomassie Blue-stained gel also showed a dose-dependent decrease of a band with the electrophoretic characteristics of protein 4.1. As Fig. 5 B illustrates, an immunoblot prepared with rabbit anti-protein 4.1 IgG confirmed a dose-dependent decrease in protein 4.1. The findings of membrane instability and quantitative decreases in two membrane skeletal proteins imply that normal membrane stability requires a normal complement of intact spectrin and protein 4.1.

Effect of Agents Altering Sulfhydryl Group Associations

To evaluate the effect on membrane properties of altering the state of thiol groups, we treated resealed membranes with the

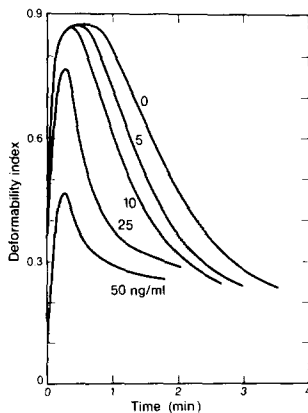


Figure 3. Effect of trypsin on membrane stability. The DI, measured at the constant applied shear stress of 575 dynes/cm², is plotted as a continuous function of time. The membranes treated with 5 ng/ml had 0.77 times the normal stability while those treated with 10 and 25 ng/ml had 0.67 and 0.42 times the normal stability.

sulfhydryl blocking agent NEM. One of the known effects of NEM is to alter the spectrin dimer-tetramer equilibrium (28). As Fig. 6 shows, NEM induces changes in membrane stability that are both concentration- and time-dependent. At low concentrations (0.1 mM) membrane stability increased over the first minute to 1.3 times normal and then decreased. After 60 min of incubation, the stability had decreased to ~80% of normal. Increasing the concentration of NEM (up to 5 mM) resulted in a similar increase in membrane stability over the first minute but a more rapid decline in stability with time so that after 5 min, the membrane stability of the 5 mM sample was decreased 10-fold. When these incubations were performed at 4°C, only the initial increase in stability was observed and the subsequent decrease which occurred at 37°C was not seen. On the other hand, NEM-induced changes in membrane deformability were not time- or temperature-dependent. Treatment with 1–5 mM NEM for 30 s resulted in a twofold decrease in deformability. The membrane deformability did not further decrease significantly over a 30-min period, and the degree of change was the same at both 4° and 37°C.

The temperature- and time-dependent alterations in mem-

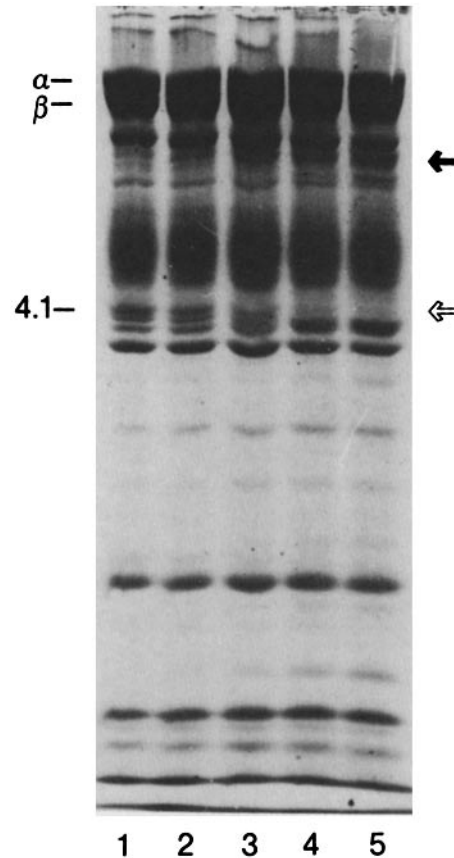


Figure 4. Effect of trypsin on membrane proteins. Membranes were treated with different concentrations of trypsin and then analyzed by SDS PAGE stained with Coomassie Blue. Lanes 1–5, membranes exposed to 0, 2.5, 5.0, 10, and 25 ng/ml of trypsin, respectively. With increasing concentrations of trypsin, there is the appearance of several new bands of molecular weight less than β -spectrin. In addition, there is a dose-dependent decrease of a band with the electrophoretic characteristics of protein 4.1. The band indicated by the closed arrow corresponds to the band in Fig. 5 A. The band indicated by the open arrow corresponds to the band in Fig. 5 B.

brane stability suggest that the effect of NEM may be a complex process. Alterations in the spectrin dimer-tetramer equilibrium may account for the observed decrease in stability, but additional changes induced by thiol blockade most likely are responsible for the initial increases in stability and for the decreases in deformability. The fact that there is no progression of the effects at 4°C suggests that the initial increase in stability and the decrease in deformability may be induced secondary to the blockade of thiol groups on cysteine residues in the exoplasmic domains of integral membrane proteins.

Effect of Membrane Protein Cross-Linking

To evaluate the effect of oxidative cross-linking of membrane proteins we treated resealed membranes with diamide, an agent known to oxidize sulfhydryl groups (11). Fig. 7 shows the effect of 0.1 mM diamide on membrane deformability and stability. In this figure, the relative stability and deformability of membranes is plotted as a continuous function of time with the stability data depicted in the top curve and the deformability data in the bottom curve. The untreated, control membranes manifested constant deformability and stability over 60 min. Treatment with 0.1 mM diamide resulted in an increase in stability and a marked decrease in deformability

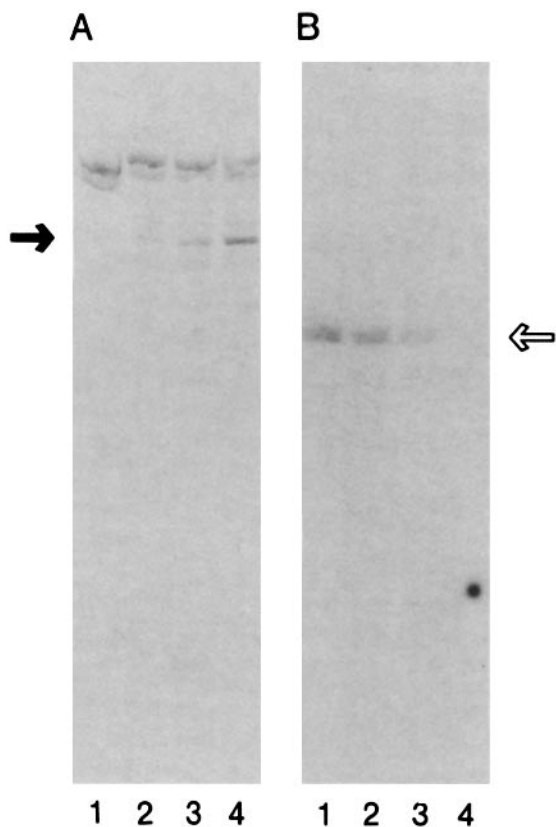


Figure 5. Western blot analysis of membranes treated with trypsin. Membranes were treated with different concentrations of trypsin and then analyzed by immunoblot. Lanes 1-4, membranes exposed to 0, 2.5, 5.0, and 10 ng/ml of trypsin, respectively. *A* was incubated with rabbit anti-spectrin IgG and *B* with rabbit anti-protein 4.1 IgG. It can be seen that with increasing concentrations of trypsin treatment, there is increasing proteolysis of spectrin (*A*, closed arrow) and a decrease in the membrane content of intact protein 4.1 (*B*, open arrow).

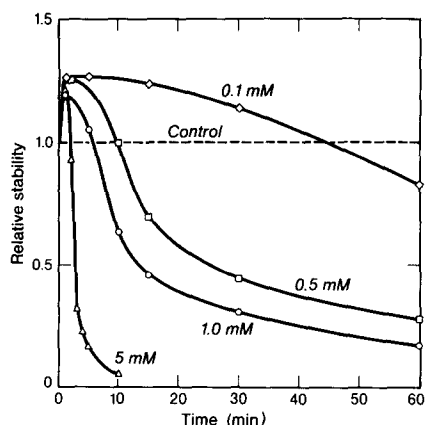


Figure 6. The effect of NEM on membrane stability. The relative stability of membranes at 37°C is plotted as a continuous function of time. The stability of the control, untreated membranes remains constant over 60 min and is depicted by the dashed line. NEM-treated membranes showed an increase in membrane stability over the first minute and then a decline in stability with time. This decline in stability was concentration-dependent.

as a function of time. With increasing concentrations of diamide up to 5 mM, both the deformability and stability changes were more pronounced and occurred at a faster rate.

Treatment of membranes with malonyldialdehyde, an amino group cross-linking reagent, also resulted in a dose-dependent increase in stability and decrease in deformability.

Membrane Deformability and Stability in Pathologic Red Cells

We next performed a series of experiments using pathologic red cells with previously defined abnormalities in skeletal protein interactions. For these studies we made membranes from the cells of a family with hereditary elliptocytosis

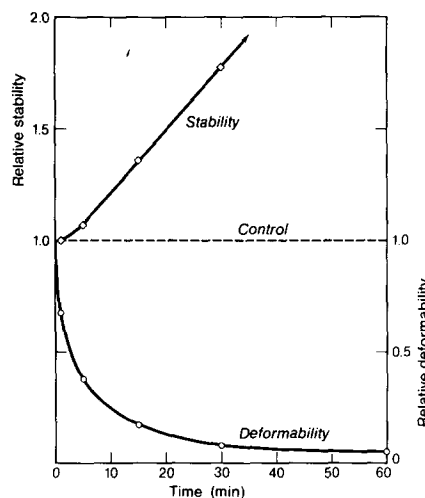


Figure 7. The effect of 0.1 mM diamide on membrane deformability and stability. The relative stability and deformability of membranes is plotted as a continuous function of time. The relative stability data are depicted in the top curve and the relative deformability data are depicted in the bottom curve. Both the deformability and stability of the control, untreated membranes remain constant over 60 min and are shown by the dashed line. Diamide treatment resulted in an increase in stability and a decrease in deformability as a function of time.

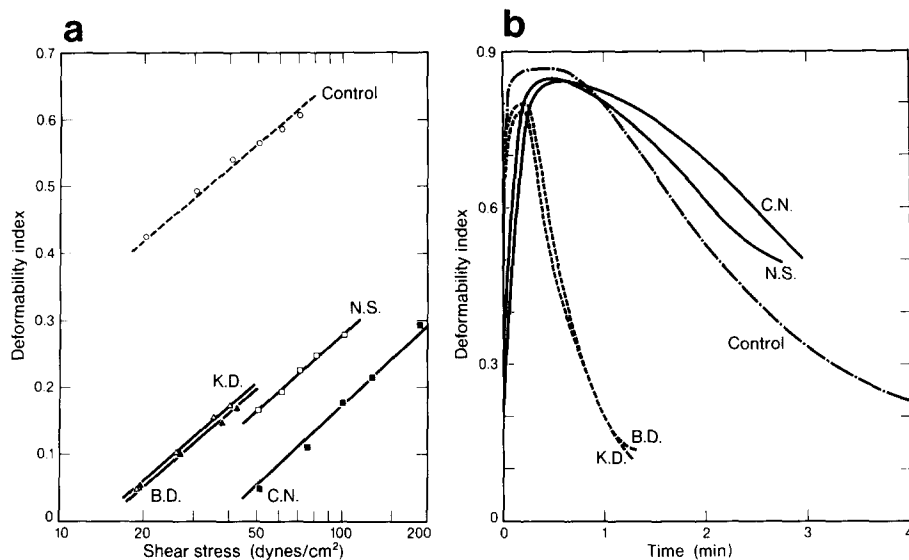


Figure 8. Membrane deformability and stability in H. E. and Malaysian ovalocytosis. Membrane deformability is depicted in *a*. *K.D.* and *B.D.*, elliptocytic membranes that had 0.1 times the normal deformability. *N.S.* and *C.N.*, membranes from ovalocytes that showed, respectively, 0.09 and 0.05 times the normal deformability. The stability of these same membranes is depicted in *b*. Elliptocytic membranes *K.D.* and *B.D.* had 0.26 and 0.28 times the normal stability. Ovalocytic membranes *C.N.* and *N.S.* showed 1.5 and 1.3 times the normal stability.

(H. E.) whose membranes had previously been shown to have an increased spectrin dimer-to-tetramer ratio (19). As Fig. 8 illustrates, unmodified H. E. membranes showed 0.1 times the normal deformability and 0.26–0.28 times the normal stability. Membranes from individuals with Malaysian ovalocytosis, as yet an uncharacterized membrane defect, also had markedly decreased deformability. These membranes had 0.05–0.09 times the normal deformability. But, in contrast to the H. E. membranes, these rigid membranes had 1.3–1.5 times the normal stability (Fig. 8). Studies of membranes from a family with an uncommon form of hereditary spherocytosis and a spectrin defect resulting in decreased binding of spectrin to protein 4.1 (34) showed essentially normal deformability while membrane stability was 0.45–0.59 times normal (Fig. 9). Finally, studies were done on membranes from individuals with hemoglobin CC, a disorder in which the primary defect is an abnormal hemoglobin and secondary skeletal changes are presumed to occur. As Fig. 9 illustrates, these membranes had 0.29–0.40 times the normal deformability and a 1.5-fold increase in stability.

Discussion

Our results show that in biochemically perturbed normal cells and in pathologic cells, the membrane properties of deformability and stability do not change in a predictable pattern in relationship to one another and therefore appear to be regulated by different skeletal protein interactions. Fig. 10 shows a model to conceptualize how these properties might be regulated by the skeletal proteins. This model is an extension of ones proposed by Waugh (31) and Shen (27), and takes into account the known structure, associations, and stoichiometry of the skeletal proteins. In the nondeformed state (*a*), the spectrin molecules exist in a folded confirmation. Reversible deformation of the erythrocyte membrane occurs with a change in geometric shape but at a constant surface area (from *a* to *b* to *c*). During reversible deformation, a rearrangement of the skeletal network occurs in which certain spectrin molecules become uncoiled and extended while others assume a more compressed and folded form. With increased shear stress, the membrane becomes increas-

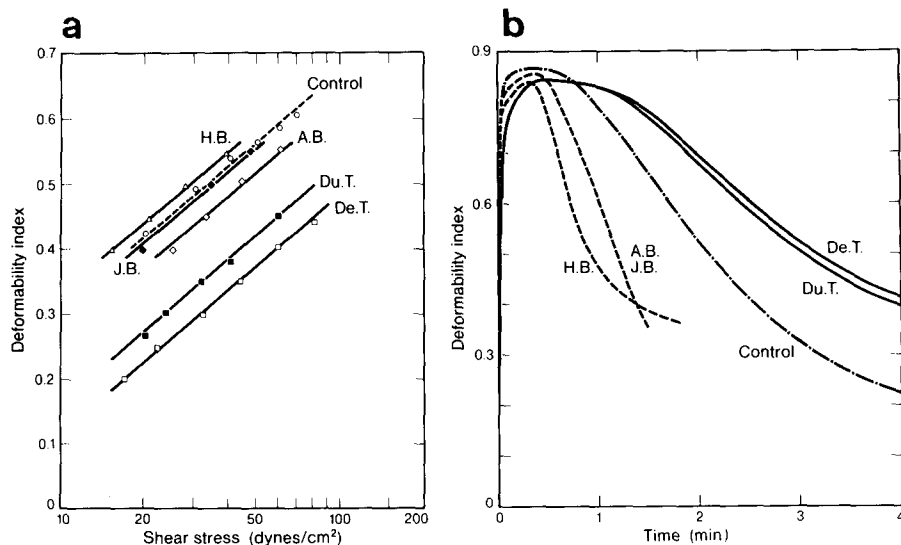


Figure 9. Membrane deformability and stability in hereditary spherocytosis and hemoglobin CC. Membrane deformability is depicted in *a*. *H.B.*, *J.B.*, and *A.B.*, spherocytic membranes showing essentially normal deformability. *Du.T.* and *De.T.*, membranes from cells with hemoglobin CC had, respectively, 0.40 and 0.29 times the normal deformability. The stability of these membrane preparations is shown in *b*. *H.B.* membranes had 0.45 times the normal stability and *J.B.* and *A.B.* membranes both had 0.59 times the normal stability. In contrast, *De.T.* and *Du.T.* membranes showed, respectively, 1.5 and 1.4 times the normal stability.

ingly extended (*b* and *c*) and some of the spectrin molecules attain their maximal linear extension. This point is the limit of reversible deformability. A continued application of force would necessitate an increase in surface area and the breaking of junction points. When the cells are exposed to a shear stress great enough to require an increase in surface area, the membrane fails. We believe that this failure occurs at the points of spectrin-spectrin or spectrin-actin-protein 4.1 association and results in fragmentation of the membrane.

The membrane skeleton can thus be viewed as a network of folded spectrin molecules held together by protein-protein associations that occur at both ends of the spectrin heterodimer. Disruption of either the spectrin-actin-protein 4.1 complex at one end of spectrin or of spectrin self-association at the other end would have a markedly detrimental effect on membrane stability. On the other hand, for the membrane to deform normally, the skeletal network must be able to undergo rearrangement and the spectrin molecules fold and unfold. Hence an increase in intermolecular or intramolecular associations of the skeletal proteins or an increased association of integral membrane proteins with the skeletal network would have a profound effect on membrane deformability.

To validate this model, we needed assays to measure quantitatively membrane properties of red cells. Quantitative measurement of membrane deformability entails determining the extent of deformation as a continuous function of

applied stress. The ektacytometric membrane deformability assay directly provides this information. In contrast, measurement of membrane stability entails determining the extent of membrane failure at defined values of shear stress. Such a fragmentation should involve disruption of protein-protein associations in the skeletal network and not the release of protein-depleted lipid vesicles. If the membrane stability assay used in the present study is actually measuring failure of protein junctions, then one would expect to see skeletal proteins present in the fragments generated by the high shear stress. The SDS PAGE analysis did indeed show that the protein contents of the smallest fragments were identical to the protein contents of the intact, resealed membranes. These findings, in addition to supporting the model, suggest that the stability assay has physiological relevance to *in vivo* processes in certain hereditary hemolytic anemias. *In vivo* in these anemias and *in vitro* in the stability assay, shear stress results in the formation of membrane protein-containing fragments with the resultant loss of surface area of the original membranes thereby converting them to non-deformable spheres.

The prediction from our model of reversible deformation and membrane failure would be that a decrease in junction associations would result in decreased membrane stability. The junction involving spectrin, actin, and protein 4.1 was altered in the 2,3-DPG-treated cells and in the hereditary

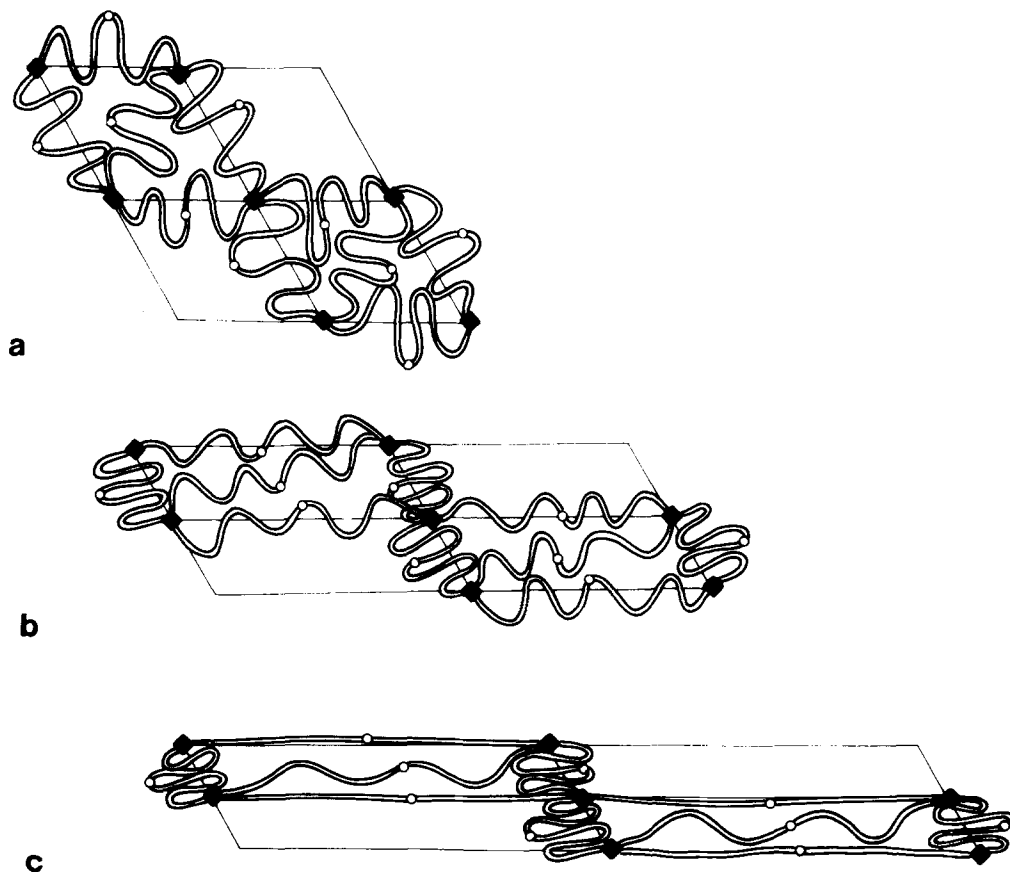


Figure 10. Model of reversible deformation of erythrocyte membrane. Reversible deformation occurs with a change in geometric shape but at a constant surface area. (*a*) The nondeformed membrane. With increased shear stress, the membrane becomes increasingly extended (*b* and *c*). Further extension of the membrane beyond that shown in *c* would result in an increase in surface area and the breaking of junction points. This is the stage at which membrane fragmentation occurs. ◆, protein 4.1, actin, and spectrin association points; ○, spectrin-spectrin association points; linear coils, spectrin dimer.

spherocytosis cells. In both these cases, we found that membrane stability was markedly decreased. It is interesting to note that 2,3-DPG-induced membrane instability is a reversible process and this finding supports earlier conclusions that this polyphosphate disrupts skeletal protein associations reversibly and does not cause irreversible changes in protein structure.

Decreased membrane stability was also noted in the NEM-treated normal cells and in the H. E. cells in which decreased spectrin-spectrin interaction is thought to be the primary defect (19). Further support for the role of spectrin self-association in membrane stability was presented in a recent report that showed a quantitative relationship between the state of spectrin oligomerization and membrane stability (15). In these studies, increases in the spectrin dimer content of the membrane correlated directly with decreases in membrane stability and decreases in spectrin tetramers.

From our model of the membrane response to shear stress, one might predict that the creation of additional protein-protein associations would result in membranes with decreased deformability. Indeed, treatment with malonyldialdehyde and diamide, two agents known to cause protein-protein cross-linking, resulted in a less deformable membrane. These membranes may well be less deformable because the oxidative cross-links between spectrin molecules would limit the extent to which they could fold or unfold at a given shear stress. Another possible mechanism by which deformability can be decreased is by increasing the number of integral protein links to skeletal proteins. An example of this mechanism is the ligand-induced association of the integral protein glycophorin A with the membrane skeletal proteins (6).

Taken together, the results of these *in vitro* studies show that the skeletal proteins play a crucial role in determining both membrane stability and membrane deformability since altering the normal state of these proteins results in changes in both of these membrane physical properties. Further, the data strongly suggest that different skeletal protein interactions regulate deformability and stability. Using the model we have presented, it should be possible to obtain a mechanistic understanding of both normal and altered red cell membrane properties and to determine which proteins, or their interactions, would be most fruitful to explore biochemically.

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