The red cell membrane derives its elasticity and resistance to mechanical stresses from the membrane skeleton, a network composed of spectrin tetramers. These are formed by the head-to-head association of pairs of heterodimers attached at their ends to junctional complexes of several proteins. Here we examine the dynamics of the spectrin dimer-dimer association in the intact membrane. We show that univalent fragments of spectrin, containing the dimer self-association site, will bind to spectrin on the membrane and thereby disrupt the connectivity of the protein network. This results in impairment of the mechanical stability of the membrane. When, moreover, the cells are subjected to a continuous low level of shear, even at room temperature, the incorporation of the fragments and the consequent destabilization of the membrane are greatly accentuated. It follows that a modest shear force, well below that experienced by the red cell in the circulation, is sufficient to sever dimer-dimer links in the network. Our results imply 1) that the membrane accommodates the enormous distortions imposed on it during the passage of the cell through the microvasculature by means of local dissociation of spectrin tetramers to dimers, 2) that the network in situ is in a dynamic state and undergoes a “breathing” action of tetramer dissociation and re-formation.

Cells that are required to withstand high mechanical stresses rely for their capacity to accommodate to distortion without structural damage on a membrane-associated complex of proteins. The archetypal example is the red cell membrane, which is subject to large shearing forces throughout its lifetime in the circulation, and responds to these by elastic deformation. The lipid bilayer itself is essentially devoid of elasticity and a protein-free bilayer membrane rapidly vesiculates under even mild shear stress. The red cell membrane skeleton, which gives the membrane its characteristic mechanical properties, is a roughly hexagonal lattice, composed of spectrin tetramers attached at their ends to junctional complexes consisting of several globular proteins (1). The spectrin tetramers are formed by the head-to-head association of pairs of αβ heterodimers. The self-association of such dimers in free solution is weak ($K_a ~3 \times 10^2 \text{ m}^{-1}$ at physiological temperature and ionic strength) (2, 3), but the apposition of the association sites on the immobilized proteins in situ ensures that the spectrin remains overwhelmingly in the tetrameric state (some higher association states, especially the hexamer, appear also to exist in the network (4)). We know little, however, about the dynamics of the spectrin dimer-dimer interaction in the intact red cell membrane; more especially, the possible effects of membrane deformation on this interaction have not been considered.

The origins of the elastic properties of the membrane remain a matter of debate. The end-to-end distance of the spectrin tetramers is constrained by the separation of the junction points to about half the equilibrium root-mean-square end-to-end distance of the protein in solution (5, 6), and this circumstance gave rise to the conjecture that the spectrin behaves as an entropy spring. More recent evidence, however, implied that this was not the predominant source of the elasticity (7), and there is indeed some structural evidence to suggest that spectrin may act as a helical Hookean spring (8). The maximum permitted local extension of the membrane, in the absence of unfolding of spectrin secondary structure (9), should be equivalent to the difference between the average separation of the junctions and the contour length of the tetramers, which is known from electron microscopy (10). This amount to an extension of about 3-fold, sufficient to explain the large distortions that the cell undergoes in vivo (11, 12) and which can be simulated in vitro (13). There are currently, however, no experimental data to discriminate between these or other kinds of local changes accompanying distortions of the membrane.

Here we show that under physiological conditions spectrin tetramers in the unstressed intact membrane exist in rapid equilibrium with dimers. Importantly, shear-induced membrane deformation markedly displaces the equilibrium in favor of the dimer. Based on these findings we suggest that such dissociation of spectrin tetramers is a primary part of the mechanism by which the membrane can accommodate the large reversible distortions that it suffers in the circulation. Such perturbation of protein-protein interactions under the action of external forces may be a general phenomenon.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human venous blood was drawn, with informed consent, from healthy volunteers. Glutathione-Sepharose 4B was purchased from Amersham Biosciences, Dextran T40 from Amersham Biosciences AB (Uppsala, Sweden), electrophoresis reagents from Bio-Rad, and GelCode Blue Reagent from Pierce. All other chemicals were reagent grade and obtained from commercial sources.

**Methods**

**Preparation of Recombinant Spectrin Fragments**—Fragments of the N-terminal region of human α-spectrin, comprising residues 1–50 and...
peptide to the spectrin on the membrane, with and without a shearing step, was analyzed by re-lysing the ghosts with 30 volumes of ice-cold Buffer A, washing three times with the same buffer, and extracting the spectrin. Extraction was accomplished by suspension of the ghosts in 0.25 mM sodium phosphate, pH 7.4, and dialysis at 4 °C overnight. The spectrin was collected by centrifugation (21,000 g) and examined by gel electrophoresis in the native state in a Tris-Bicine buffer system, run in the cold (16). The spectrin tetramer, dimer, and the complex of the dimer with the peptide fragment were well resolved, and the absence of a zone corresponding to the free fragment or of a trail of stained material showed that no dissociation had occurred during migration. The gels, stained with GelCode Blue, were evaluated by densitometry.

**Results**

**Binding of α-Chain Peptide Fragment to Spectrin Self-association Sites in Situ** — The dimer-tetramer equilibrium of spectrin is characterized by an unusually high activation energy (2). Thus at room temperature many hours are required to approach equilibrium, and in the cold the half-time is measured in weeks or months. The explanation of this phenomenon is that formation of the tetramer from its constituent αβ dimers through a pair of intermolecular α-β bonds requires the prior rupture of two intramolecular α-β bonds, one in each of the antiparallel dimers (3, 18). Because the intra-dimer bond has to open to allow the N-terminal α-chain fragment to bind to its C-terminal site on the β-chain of a dimer, the high activation energy persists in the fragment-dimer interaction. Therefore to approach binding equilibrium within a reasonable time the experiment must be carried out at elevated temperatures (30–37 °C) (16, 19, 20). Fig. 1A shows that at these temperatures (but not at the lower temperature of 24 °C) incorporation of the α1–154 peptide into the spectrin in the membrane network does indeed occur. (A trace of spectrin dimer is always seen in the gel; its amount varies, and it is probably a consequence, at least in part, of proteolytic damage before or during extraction (21). The peptides with and without the GST fusion domain were tested and no differences were found (data not shown). As a control, we also examined the short N-terminal α1–50 peptide, which does not enter the native fold and does not therefore bind to the β-chain in solution (14, 22); this peptide was not incorporated into the membrane (Fig. 1B). We have further shown that the long peptide does not bind to extractin and actin-free inside-out membrane vesicles (data not shown), which retain all other intrinsic and extrinsic membrane proteins. Thus any possibility that the peptide exerts its effect by binding to other proteins can be excluded.

From the kinetics of incorporation of the α1–154 peptide (Fig. 1C), it is clear that the binding is an equilibrium process,
effectively reaching completion after about 50 min at 37 °C. The absence of a tetramer-fragment complex shows that the binding of a single peptide molecule causes dissociation of the tetramer into dimers, one or probably both (since the amount of free, uncomplexed dimer generated does not significantly increase) associated with the peptide. Fig. 2 reveals that the binding of the peptide to the spectrin in situ is reversible, for when the cells with incorporated peptide were washed free of unbound peptide and warmed to 37 °C, the peptide was released from the membrane. Slower release ensued at 30 °C, but none could be detected at 24 °C over a period of 40 min.

**Effect of Peptide Incorporation on Membrane Stability**—The effect of increasing concentrations of the long peptide on the mechanical stability of the membrane was assessed by shearing in the ektacytometer at room temperature (15). As Fig. 3A shows, membrane stability is markedly reduced in ghosts containing the peptide, as reflected by a faster rate of decay of the DI. Increasing concentrations of the peptide in the resealing buffer resulted in a progressive decrease in membrane mechanical stability. The peptides with and without GST fusion domain were again tested and no differences were found. The decreased membrane mechanical stability was paralleled by a progressive increase in the incorporation of the peptide into the membrane skeleton (Fig. 3B). Fig. 3C shows that increasing concentrations of peptide in the resealing buffer led to a progressive accretion of the spectrin dimer-peptide complex, with a corresponding decrease in the concentration of spectrin tetramers. In Fig. 3D we show the impairment of membrane stability, measured by the half-time of breakdown under shear as a function of the extent of tetramer dissociation. A similar relationship between decreased membrane mechanical stability and elevated dimer content has previously been observed in red cells of subjects with hemolytic anemias, caused by spectrin mutations that result in defective dimer self-association (23). The short N-terminal α1–50 peptide, which did not incorporate into the membrane, had no effect on membrane mechanical stability at concentrations up to 100 μM in the resealing buffer (data not shown).

**Incorporation of the α-Chain Peptide into the Membrane under Shear**—Resealed ghosts containing the α1–154 peptide were subjected to varying periods of low shear in the ektacytometer at room temperature. Because of the high activation energy of the binding and tetramer dissociation reactions, there is, on the time scale of these experiments, no detectable incorporation of the peptide into the membrane network in static cells (or of course binding to spectrin in free solution).
Nevertheless, a time-dependent incorporation of the peptide was observed (Fig. 4). This striking effect demonstrates that mild shearing stress is sufficient to induce dissociation (presumably local) of spectrin tetramers, thus overcoming an activation energy of some 100 kcal mol$^{-1}$ (2).

**DISCUSSION**

While the self-association of spectrin dimers in free solution is weak, especially at physiological temperature, the cohesion of the membrane skeletal network *in situ* is ensured by the close apposition of the binding sites. The strong link- age of the distal dimer ends to the network junctions, and especially the tight attachment of one dimer in each tetramer to membrane-bound ankyrin at a position close to the self-association site (24), can be assumed to restrict severely the excluded volume available to the dimer association sites. The dimer-tetramer equilibrium *in situ* is thus expected (and observed) to be grossly shifted in favor of the tetramer on entropic grounds. Binding of the univalent fragments at the dimer-dimer association sites was thus expected, if it occurred at all, to require a very large molar excess of the fragment, as was indeed found.

The association constant for binding of a univalent α-chain fragment to spectrin dimer in dilute solution is only a little lower than that for dimer self-association (16, 19, 20). This may be because only one intra-dimer interaction has to be broken to allow the fragment to bind. In any case, the fragment would in principle be expected to bind to any available dimers on the membrane. For dimers to become available, the spectrin in the network must undergo a continuous association-dissociation, or “breathing” process of a frequency compatible with entry of the fragment. The apparent association constant for the binding of the fragment to its sites in the network *in situ* should be defined by a simple Langmuir adsorption isotherm, formally equivalent to the Scatchard equation (25). However, the concentration of available dimers depends on the *in situ* dimer-tetramer equilibrium. This is concentration-independent, since no diffusion of the reactants on the membrane is permitted. It can thus be treated as a conformational equilibrium between an open and a sequestered state of the dimers. The system is therefore defined by two equilibria: $S + F = SF$ and $S = S_F$, where $S$ and $S_F$ represent the available and sequestered states of the spectrin dimer, respectively, and $F$ the univalent fragment. Then if $K_D$ and $K_F$ are the equilibrium constants for these two reactions, and writing $a$ for the fractional saturation of spectrin on the membrane with the fragment (expressing spectrin concentration in molar units of dimers), the binding of the fragment to the membrane is described by the relation: $a = KK_D/ff(1f + 1)$, where $f$ is the concentration of the fragment. This equation was used to fit the data points of Fig. 3C and gave a value for $K' = KK_D$ of $1.5 \times 10^4$ m$^{-1}$. To extract $K_D$, we need to know $K_D$, but its value for the interaction in free solution cannot be equated with that for binding on the membrane, for it may well be grossly influenced by steric and electrostatic factors. A value in the range of those obtained from solution studies (16, 19, 20), say $10^4$ m$^{-1}$, would lead to $K_D$ in the region of 0.015; that is about 1% of the tetramer population would be dissociated in the unperturbed cell. This proportion would almost certainly be further reduced by molecular crowding caused by hemoglobin (26). A more soundly based estimate must await a direct experimental determination of $K_D$.

The most striking outcome of this study is the observation that the dissociation of tetramers into dimers can be induced by shear, even at room temperature at which the equilibrium in solution is essentially frozen over the period of the experiment. This implies that a very modest mechanical force is sufficient to break the dimer-dimer interaction. It also implies that this association-dissociation, or breathing process operates continuously in the circulation, in which the cells are nearly always under shear. Our data do not as yet permit a rigorous quantitative description of this previously unsuspected effect. The influence of the membrane environment on the equilibrium and rate constants for the self-association of spectrin dimers and the interaction of spectrin dimers with a univalent fragment, as measured in dilute solution, is still uncertain. We can also not exclude that some additional dissociation of spectrin tetramers through entry of more peptide into the spectrin network could have occurred during the brief period of the high-shear assay at room temperature. Thus the fractional dissociations of tetramers engendering the observed reductions in membrane stability should be regarded as minimum values. The likelihood of dissociation of bound peptide during the time of experimental manipulations after the cells were restored to the static state is remote. Various explanations have been advanced for the elasticity and stability of the membrane. One type of model is based on the stretching of spectrin from its relatively crumpled (27) or compressed (8) state at rest up to its fully extended length (but see also Ref. 7), allowing for an extension factor of about 3. Another suggestion is that the secondary structure of the protein, which is composed primarily of three-stranded α-helical elements (28), can be unfolded under the action of a tensile force (9). The question of whether protein-protein interactions in the network can be disrupted by mechanical forces has not previously been addressed. It is unlikely that dissociation would occur at the lattice junctions, for the ternary complex of spectrin, actin, and protein 4.1 (irrespective of contributions of other proteins present at the junctions) is very tightly associated (29). The results presented here indicate that rupture of spectrin tetramers is a likely mechanism for the capacity of the membrane to adapt to very large distortions.

These observations offer a rationale for the evolutionary advantage of the tetrameric structure of spectrin. If it functioned only as a simple elastic element of the network a fragile dimer-dimer link at the center would afford no advantage. It may be recalled that neuronal spectrin, fodrin, which is probably not exposed to high shearing forces during its lifetime in the cell, has the form of a stable tetramer, which cannot be dissociated into dimers by known physical means, short of denaturation (30).

**REFERENCES**

1. Mohandas, N., and Evans, E. (1994) *Annu. Rev. Biophys. Biomol. Struct.* 23, 787–814
2. Ungewickell, E., and Gratzer, W. (1978) *J. Eur. Biochem.* 89, 379–385
3. Henniker, A., and Ralston, G. B. (1994) *Biophys. Chem.* 52, 251–258
4. Morrow, J. S., and Marchesi, V. T. (1981) *J. Cell Biol.* 88, 463–468
5. Stokke, B. T., and Elgsaeter, A. (1981) *Biochim. Biophys. Acta* 640, 640–645
6. Reich, M. H., Kam, Z., Eisenberg, H., Worster, D., Ungewickell, E., and Gratzer, W. B. (1982) *Biophys. Chem.* 16, 307–316
7. Hansen, J. C., Skalak, R., Chien, S., and Hoger, A. (1996) *Biophys. J.* 70, 146–166
8. McGough, A. M., and Josephs, R. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 5208–5212
9. Rief, M., Pascual, J., Saraste, M., and Gaub, H. E. (1999) *J. Mol. Biol.* 286, 553–561
10. Shotton, D., Burke, B., and Branton, D. (1979) *J. Mol. Biol.* 131, 303–329
11. Skalak, R., and Brummerken, P. F. (1969) *Science* 164, 717–719
12. Mohandas, N., and Chasis JA. (1993) *Biophys. Chem.* 46, 164–187
13. Disher, D. E., and Mohandas, N. (1996) *Biophys. J.* 71, 1680–1694
14. Nicolas, G., Pedroni, S., Fournier, C., Gauthier, H., Cras, M., and Leconte, L. M. (1998) *Biochem. J.* 332, 81–89
15. Mohandas, N., Clark, M. R., Health, B. P., Ross, M., Wolfe, L. C., Lux, S. E., and Shohet, S. B. (1992) *Blood* 80, 768–774
16. Shahbakhsh, F., and Gratzer, W. B. (1986) *Biochemistry* 25, 5969–5975
17. Danilov, Y. N., Fennell, R., Leng, E., and Cohen, C. M. (1990) *J. Biol. Chem.* 265, 2556–2562
18. Delito, T. M., K-C. Peng, Speicher, K. D., and Speicher, D. W. (1992) *Biochemistry* 31, 10872–10878
19. Morrow, J. S., Speicher, D. W., Knowles, W. J., Hau, C. J., and Marchesi, V. T. (1890) *Proc. Natl. Acad. Sci. U.S.A.* 77, 6952–6966
20. Hanspal, M., and Ralston, G. B. (1982) Biochim. Biophys. Acta 709, 105–109
21. Backman, L., Pekrun, A., and Gratzer, W. B. (1991) J. Biol. Chem. 266, 3835–3840
22. Lecomte, M. C., Nicolas, G., Dhermy, D., Pinder, J. C., and Gratzer, W. B. (1999) Eur. Biophys. J. 28, 208–215
23. Lane, P. A., Shew, R. L., Iarocci, T. A., Mohandas, N., Hays, T., and Mentzer, W. C. (1987) J. Clin. Invest. 79, 989–996
24. Bennett, V., and Gilligan, DM. (1993) Annu. Rev. Cell Biol. 9, 27–66
25. Klotz, I. M. (1985) Quart. Rev. Biophys. 18, 227–259
26. Liu, S.-C., and Palek, J. (1984) J. Biol. Chem. 259, 11556–11562
27. Versteeg, B. G., and Steck, T. L. (1989) Biophys. J. 55, 253–262
28. Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C., and Branton, D. (1993) Science 262, 2027–2030
29. Ohanian, V., Wolfe, L. C., John, K. M., Pinder, J. C., Lux, S. E., and Gratzer, W. B. (1984) Biochemistry 23, 4416–4420
30. Davis, J., and Bennett, V. (1983) J. Biol. Chem. 258, 7757–7766