Dependence of the Permanent Deformation of Red Blood Cell Membranes on Spectrin Dimer–Tetramer Equilibrium: Implication for Permanent Membrane Deformation of Irreversibly Sickled Cells

By Shih-Chun Liu, Laura H. Derick, and Jiri Palek

Red blood cells (RBCs) in sickle cell anemia, transformed into a sickled shape by prolonged deoxygenation, or normal RBCs deformed by a prolonged micropipette aspiration become permanently stabilized in their abnormal shape. This semisolid plastic behavior is thought to involve an irreversible reorganization of the membrane skeleton, but the exact nature of this skeletal rearrangement is not known. In this study, we first asked whether the irreversible deformation is associated with a permanent stretching of the skeletal network, and then whether it is due to a rearrangement of skeletal components involving a disruption of pre-existing protein associations and the subsequent reassociation of new protein contacts. Having found no ultrastructural evidence of stretching of the skeletal lattice in membranes derived from permanently deformed RBCs, we addressed the possibility of reorganization of the proteins of the membrane skeleton. We examined the temperature dependence of irreversible cell deformation to see if it correlated with the known temperature dependence of spectrin tetramers to dimer dissociation and reassociation. Testing the shape irreversibility of both deoxygenated reversibly sickled cells and Nucleopore-aspirated normal cells, we found that both types of cells became permanently deformed when the prolonged incubation of applied force or deoxygenation was performed at 37°C, the temperature at which spectrin tetramers were free to dissociate and reassociate. In contrast, both types of cells were able to regain their original discocytic shape if the prolonged incubation was performed at the lower temperature: at less than 13°C instead of 37°C. Furthermore, normal RBCs were incubated with inosine and pyruvate to elevate intracellular 2,3-diphosphoglycerate, the polyanion shown to destabilize spectrin–actin–protein 4.1 association. This did not result in a promotion of irreversible deformation of these cells. We conclude that the irreversible cell deformation observed at physiologic temperature is associated with a skeletal rearrangement through dissociation of spectrin tetramers to dimers and a subsequent reassociation of dimers to tetramers in the new (deformed) configuration. These findings may explain a permanent stabilization of irreversibly sickled cells in their abnormal shape in vivo.

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glyceraldehyde (2,3-DPG), the polyanion known to destabilize spectrin–actin–protein 4.1 association.\textsuperscript{12,18}

**MATERIALS AND METHODS**

**Negative-staining electron microscopy.** Blood from patients with homozygous sickle cell disease was collected in acid–citrate–dextrose anticoagulant tube; RBCs were sedimented at 2,000 rpm for 10 minutes and washed with phosphate-buffered saline (PBS) and fractionated in a discontinuous stractan gradient with a density range of 1.128 to 1.158 g/mL.\textsuperscript{19} The densest cell fraction (1.158 g/mL) containing greater than 90% ISCs was collected, washed, and lysed hypotonically to obtain ISC ghosts for ultrastructural study.

Ghosts were examined by negative-staining electron microscopy as previously described.\textsuperscript{20} Briefly, the ghosts were applied to carbon-coated grids, rinsed with low salt buffer (0.1 mmol/L NaPi, pH 8.0), and negatively stained with uranyl acetate solution. The excess solution was drawn into filter paper and grids were air dried. The double-layered skeleton networks in these spicules of the flattened ghosts were visualized in a JEOL (Tokyo, Japan) JEM-100S electron microscope with an accelerating voltage of 60 kV.

**Temperature-dependent irreversible sickling.** Reversible sickle cells (RSCs), obtained in the light-density fraction (1.09 to 1.128 g/mL interface) after stractan gradient centrifugation, were resuspended at 5% hematocrit in buffer containing 20 mmol/L HEPES (pH 7.4), 2 g/L glucose, 0.54 mmol/L adenine, 12.7 mmol/L inosine, 2 mmol/L MgCl₂ and 120 mmol/L KCl. The suspension was incubated under N₂ at 37°C for 1 hour to induce cell sickling. Subsequently, the cell suspension was split into two portions: one incubated at 37°C and the other at 13°C under N₂. After a total of 24 hours of incubation, a small aliquot from each suspension was aspirated into a syringe (1 mL) containing deoxygenated glutaraldehyde (2.5%). The rest were reoxgenated for 1 hour under air. The morphology of these cells was examined by phase contrast light microscopy. Some of the reoxgenated cells were further incubated under carbon monoxide at 25°C for 15 minutes before microscopic examination.

**Temperature-dependent spectrin tetramer = dimer transformation in solution.** Crude spectrin enriched in spectrin tetramers or spectrin dimers was prepared by low-salt (0.1 mmol/L NaPi, pH 8.0) extraction of RBC ghosts at 0°C for 16 hours or at 37°C for 20 minutes, respectively. These crude extracts (2 mg protein/mL) were incubated for 24 hours at 13°C or 30°C in isotonic buffer containing 5 mmol/L NaPi (pH 7.4), 150 mmol/L NaCl, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and analyzed for spectrin conversion by nondenaturing gel electrophoresis and densitometry as previously described.\textsuperscript{21}

**Permanent skeletal deformation of normal RBCs induced by Nuclepore filter aspiration.** Fresh normal RBCs were washed and resuspended in ATP-maintaining buffer containing 20 mmol/L HEPES (pH 7.4), 0.54 mmol/L adenine, 12.7 mmol/L inosine, 1% bovine serum albumin, 2 mmol/L MgCl₂, 2 g/L glucose, and 110 mmol/L KCl to obtain 290 to 300 mOsm. Cells in the suspension were aspirated into a polycarbonate filter (Nuclepore, Pleasanton, CA) with a small pore diameter (0.6 to 1.0 μm) and subjected to a constant hydrostatic pressure of 6 cm water on the RBCs across the filter. After brief (10 seconds) and prolonged (18 hours) aspiration at 37°C, RBCs on the Nuclepore filter were released by reversing the pressure. The detached cells were fixed with glutaraldehyde (1%). Membrane skeletons (Triton shells) were prepared from RBC ghosts of aspirated RBCs by Triton X-100 (1% v/v) extraction in 5 mmol/L NaPi (pH 7.4) at 0°C. RBCs, ghosts, and skeletons were examined without staining under a light microscope with a contrast enhancement Newvicon camera (MT1 Inc, Michigan City, IN) connected directly to a video monitor.

**Temperature-dependent irreversible deformation of normal RBCs by prolonged aspiration.** Normal RBCs in ATP-maintaining buffer were subjected to aspiration with a Nuclepore filter (1 μm pore diameter) at two different temperatures (37°C and 4°C). A constant hydrostatic pressure of 6 cm water was applied across the filter. After aspiration for 18 hours, RBCs were released from the Nuclepore filter, incubated at 37°C for 30 minutes, and examined by light microscopy for the presence or absence of permanent cell deformation.

**Effect of intracellular levels of 2,3-DPG on irreversible deformation of normal RBCs.** Fresh normal RBCs were treated with sodium azide and followed by incubation for 18 hours with inosine and pyruvate as previously described.\textsuperscript{22} The levels of intracellular 2,3-DPG were measured according to the published procedure.\textsuperscript{23}

**RESULTS**

**Absence of spectrin stretching in the elongated spicules of ISC ghosts.** Examination of the double-layered membranes of ISC ghosts by negative-staining electron microscopy showed a filamentous reticulum made of irregular wavy structures 7 to 12 nm in diameter (Fig 1a and b). The reticulum was uniformly distributed throughout the ISC ghost membrane (Fig 1b). We did not detect any signs of spectrin stretching in the elongated spicules of ISC ghosts. This is in contrast to artificial extension of normal RBC membranes during the air drying process in which the fiberlike structures of spectrin are stretched and aligned parallel to the direction of the extension (Fig 1c and d). Thus, the ISC deformation does not appear to involve a permanent stretching of spectrin filaments in the skeleton along the long axis of the deformation.

**Temperature dependence of the irreversible deformation of sickle cells on prolonged anaerobic incubation.** Using stractan gradient centrifugation, we obtained a light density cell fraction (1.09 to 1.128 g/mL interface) free of ISCs. These discocytic cells (RSCs) were incubated under N₂ for an hour to induce sickling. The isotonic buffer contained 120 mmol/L KCl and no NaCl to minimize the cell dehydration during prolonged incubation through action of the Na-K-ATPase. The buffer also contained adenine, inosine, glucose, and 2 mmol/L MgCl₂ to maintain the cellular ATP during the incubation. Subsequently, the cell suspension was split into two portions: one portion was incubated at 37°C (Fig 2b) and the other at 13°C (Fig 2d), both under N₂. After 24 hours of incubation, the cells were reoxgenated for 1 hour under air and examined for their shape reversal to discocytes (Fig 2c and e). We found that most of the cells subjected to a prolonged anaerobic incubation at 37°C no longer reversed to discocytic shape upon reoxygenation (Fig 2c), as reported by others.\textsuperscript{14,15} In a striking contrast, cells incubated anaerobically at 13°C reversed nearly completely to discocytic shape after reoxygenation (Fig 2e). At 13°C, the spectrin conversion was blocked to nearly the level achieved at 4°C (data not shown) but the temperature was still high enough to allow the polymerization of hemoglobin S (HbS) and maintain the sickle shape after deoxygenation (Fig 2d). As the temperature of incubation decreased from 37°C to 13°C, the measured solubility of the oxygenated HbS increased only slightly from 23.2 to 25.6 g/dL. The ISCs produced in vitro appear to have only short spicules (Fig 2c). This is due to an uncoupling of lipid bilayer from the membrane skeleton at the proximal
ends of the long spicules during sickling and a subsequent
detachment of the proximal ends from the cell body upon
reoxygenation.

The intracellular ATP levels were well maintained and
measured in a typical experiment as 1.8 mmol/L and 1.4
mmol/L in deoxygenated sickled cells after prolonged in-
cubation at 37°C and 13°C, respectively. The irreversible
sickling produced by incubation at 37°C was not due to an
incomplete depolymerization of HbS polymers upon reox-
genation, because the sickle shape distortion was also de-
tected in RBCs that were both reoxygenated and treated with
carbon monoxide, as well as in their ghosts (Table 1). To
assess whether the cell dehydration is responsible for the per-
manent cell deformation, we examined the shape reversal
after cells were incubated anaerobically (18 hours) at 13°C
in hypertonic buffer (375 mOsm). Despite the fact that the
cell volume was reduced about 9% as detected by the hand
hematocrit measurement, no permanent cell deformation was
detected after the hypertonic incubation at 13°C (data not
shown). Conversely, anaerobic incubation of sickle cells at
37°C in hypotonic buffer (250 mOsm), which increased the
cell volume by about 15%, did not prevent the cells from
becoming permanently deformed (data not shown). The ex-
tent of cell dehydration detected after prolonged incubation

RSCs, O₂

37°C,N₂, 24hrs Reoxygenated, 1 hr

13°C,N₂, 24hrs Reoxygenated, 1 hr

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Fig 1. Absence of spectrin stretching in the elongated spicules of ISC ghosts. (a and b) Membrane ghosts prepared from ISC isolated
from peripheral blood. (c and d) Normal ghosts artificially stretched during specimen preparation. Ghosts were fixed with 2.5% glutaraldehyde,
negatively stained with 1% uranyl acetate, air dried, and examined by transmission electron microscopy. The double-layered membrane
skeletal network of the flattened ghosts was preferentially stained under these conditions. No signs of spectrin stretching were detected
in the elongated spicules of ISC ghosts as shown in (b). In contrast, the stretching of fiberlike structures (apposed arrows) can be readily
seen in the artifactually extended normal ghosts induced during air drying shown in (d).

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Fig 2. Prolonged anaerobic incubation of RSCs, at 37°C, but not at 13°C, induces their irreversible sickling. RSCs were incubated under
N₂ at 37°C for 1 hour to induce cell sickling. Subsequently, the cell suspension was split into two portions: one incubated at 37°C and the
other at 13°C under N₂. After 24 hours of incubation, a small aliquot from each suspension was aspirated into a syringe (1 mL) containing
deoxygenated glutaraldehyde (2.5%). The remaining sample was reoxygenated for 1 hour under air. (a) RSCs before anaerobic incubation.
(b and c) RSCs incubated at 37°C (24 hours) under N₂ (b) and after reoxygenation (c). (d and e) RSCs incubated at 13°C (24 hours) under
N₂ (d) and after reoxygenation (e). Note that the deoxygenated RSCs incubated at 13°C, but not at 37°C, reversed to discocytes upon
reoxygenation.
SPECTRIN REARRANGEMENT IN SICKLED RBCs

Table 1. Permanent Shape Deformation of RSCs by Prolonged Deoxygenation at 37°C

<table>
<thead>
<tr>
<th>No. of Cells Studied</th>
<th>No. of Cells With Spicules</th>
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<tbody>
<tr>
<td>Deoxygenated RSCs</td>
<td>112</td>
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<tr>
<td>Reoxygenated and CO-treated RSCs</td>
<td>137</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of Ghosts Studied</th>
<th>No. of Ghosts With Spicules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane ghosts from reoxygenated and CO-treated RSCs</td>
<td>104</td>
</tr>
</tbody>
</table>

Temperature dependence of the spectrin dissociation and reassociation. The temperature-dependent inhibition of spectrin tetramer \( \Rightarrow \) dimer conversion is illustrated in Fig 3. We have prepared spectrin extracts from normal white ghosts and incubated them at 30°C or at 13°C. The conversion of spectrin tetramers to dimers was analyzed by nondenaturing gel electrophoresis. As previously reported,\(^{21,24}\) the original 0°C low-salt extract was enriched in spectrin tetramers (Fig 3a). After a 30°C incubation for 4 hours, about half of the spectrin tetramers dissociated into dimers reaching a new equilibrium (Fig 3b). In contrast, incubation of the 0°C extract at a low temperature of 13°C, which inhibited the irreversible cell deformation, produced a nearly complete inhibition of spectrin tetramer to dimer conversion (Fig 3c). In a complementary experiment using 37°C low-salt extract, which is enriched in spectrin dimers (Fig 3d), we found that spectrin reassociation took place at 30°C but not at 13°C (Fig 3e and f).

Temperature dependence of the irreversible deformation of normal RBCs on prolonged aspiration. To obtain additional support for the conclusion that the permanent deformation of sickled cells is a consequence of spectrin rearrangement, we deformed normal cells by aspiration on polycarbonate filters (Nuclepore) with an average pore diameter of 1.0 \( \mu \)m at 37°C and a hydrostatic pressure of 6 cm water. Under these conditions, tongues of membranes partially penetrated the filter (Fig 4b). If the duration of the aspiration was short (eg, 10 seconds), the cell reverted to discocytic shape after the release from the filter (Fig 4c). The membrane ghosts and skeletons derived from these cells were morphologically intact (Fig 4d through e). In contrast, if cells were aspirated for prolonged periods (eg, 18 hours), they became permanently deformed (Fig 4f). The irreversible deformation was retained in the ghosts (Fig 4g) as well as in the Triton-insoluble skeletons (Fig 4h).

To verify that the permanent deformation of normal cells by Nuclepore filter aspiration is due to spectrin rearrangement, we studied the temperature-dependent deformation of normal cells. Permanent deformation of normal cells by prolonged Nuclepore aspiration was detected at 37°C (Fig 5b), but not at 4°C (Fig 5c). It is evident that temperature affects the plastic deformation of normal cells similar to that in sickle cells.

Effect of 2,3-DPG on the irreversible deformation of normal RBCs. Because high levels of 2,3-DPG are known to destabilize spectrin–actin–protein 4.1 interaction,\(^{15,18}\) we have examined the effects of elevated concentrations of 2,3-DPG on the irreversible shape deformation. We first treated normal RBCs with sodium azide, followed by incubation with inosine and pyruvate, and then subjected the cells to prolonged aspiration (16 hours) at either 7°C or 37°C. The levels of 2,3-DPG measured were 1.6 and 10.3 mmol/L in untreated and...
the pyruvate-treated RBCs, respectively. The elevated 2,3-DPG levels did not alter the propensity of cells to undergo permanent deformation. Permanent deformation was detected only in the RBCs incubated at 37°C, but not at 7°C, regardless of the 2,3-DPG level (data not shown).

**DISCUSSION**

The objective of the present study was to elucidate the basis of permanent cell deformation of ISC or, in a model system, of RBCs permanently deformed by prolonged aspiration into a micropipette, or of sickle cells incubated under deoxygenated conditions. We hypothesized that the observed permanent cell deformation involves the dissociation and reassociation (ie, rearrangement) of membrane skeletal proteins. We examined the two possible sites of protein rearrangements, ie, spectrin dimer–dimer association, the weakest of the membrane protein interactions, and spectrin–actin–protein 4.1 association. Our results indicate that the spectrin dimer–dimer association, rather than the spectrin–actin–protein 4.1 linkage, is the primary site of skeletal rearrangement. We propose that this rearrangement involves a dissociation of the stretched spectrin tetramers into dimers and...
a subsequent formation of new bonds between the adjacent spectrin dimers stabilizing the skeleton in the altered configuration. This conclusion is based on the following observations. (1) Spectrin dimer–dimer association (kd = 4 × 10⁻⁶ mol/L) is much weaker than the spectrin–actin–protein 4.1 interaction (kd = 1 × 10⁻¹² [mol/L])². Thus, the dissociation of spectrin dimer–dimer linkages occurs more readily than that of the spectrin–actin–protein 4.1 linkages. (2) The temperature dependence of the irreversible cell deformation (Figs 2 and 5) correlates with that of the spectrin tetrmeric association (kd = dimers conversion (Fig 3), but not that of the spectrin–actin–protein 4.1 association. The low temperature (<13°C) that blocks the spectrin tetrmeric association does not inhibit the spectrin–actin–protein 4.1 interaction.³⁵⁻⁶ The propensity of irreversible deformation is not increased in RBCs enriched in 2,3-DPG, the polyanion that weakens the spectrin–actin–protein 4.1 association.

Although our data clearly indicate the role of the spectrin dimer-tetramer equilibrium in the permanent shape deformation, other factors may contribute to the permanent deformation of irreversibly sickled cells. Recent rheologic data have implicated that the plasticlike behavior of sickle membrane could also result from the association of sickle hemoglobin with the membrane in the dense, dehydrated cells.¹¹⁻¹² However, this type of plasticlike behavior was reversible upon rehydration of the cells, which decreased the association between the sickle hemoglobin and the membrane.¹¹ This is in contrast to the deformation of normal cells induced by prolonged aspiration or the deformation of sickle cells by prolonged deoxygenation: both deformations described in the present study are not reversible by cell hydration. In addition, the deformations reported here persist in the isolated ghosts and skeletons, suggesting a reorganization of the membrane skeletal proteins. The plasticlike behavior of sickle membrane has also been found to be associated with an abnormal oxidation of membrane skeletal proteins.¹³⁻³⁰ However, the observed permanent deformation induced by prolonged aspiration of normal cells or prolonged deoxygenation of sickle cells is unlikely to be caused by the membrane protein oxidation, because no significant protein crosslinking was detected in the sickle cells after prolonged deoxygenation.¹¹ Furthermore, ultrastructural examination showed no sign of stretching of spectrin filaments along the long axis of the deformed ISC ghosts (Fig 1), suggesting that the spectrin in the long spicules was rearranged rather than stretched and cross-linked.

Because the spectrin dimer–tetramer equilibrium is known to be sensitive to hemoglobin concentration,³² it is possible that dehydrom in ISC may play a role in promoting spectrin rearrangement, thus affecting indirectly the plastic flow behavior of these cells. It has been shown that a brief aspiration (in minutes) of dehydrated sickle cells by micropipette produced persistent membrane bumps.¹¹ However, this deformation was reversed once the cell was released into hypotonic medium.¹¹ Spectrin may be temporarily stretched in the briefly aspirated dehydrated sickle cell in the presence of membrane-bound hemoglobin gel, but the rearrangement of spectrin leading to an irreversible skeletal deformation requires a prolonged incubation (eg, in hours) at 37°C to generate sufficient dissociation/reassociation of spectrin.

The intracellular polymerization of HbS in deoxygenated sickle cells generates, in addition to spectrin rearrangement, another membrane damage in the long spicules, namely, the uncoupling of the lipid bilayer from the membrane skeleton.¹⁹ As visualized by immunofluorescence and immunoelectron microscopy, we have recently demonstrated that antibodies against band 3, the major lipid-associated transmembrane protein, labeled the whole cell body including the entire length of the long spicule, whereas antibodies against spectrin labeled only the cell body and the base region of the spicules. Thus, in the proximal end of the spicule of deoxygennated sickled cells, the lipid bilayer that laminates the HbS polymers is uncoupled from the membrane skeleton. Upon reoxygenation of these cells, the proximal ends of the spicules containing no spectrin become round and detach from the body, whereas the remaining base regions of the spicules, which possibly contain the rearranged spectrin, appear as short, pointed protrusions (Fig 2c).

In summary, we conclude that the permanent membrane skeletal deformation observed at physiologic temperature may be caused by a rearrangement of spectrin dimer–dimer interactions through dissociation of spectrin tetrmeric to dimers, and a subsequent reassociation of dimers to tetrmeric in the new, but deformed, configuration. These findings may explain the permanent deformation of normal cells deformed by prolonged micropipette aspiration, as well as the deformation of sickle cells induced in vitro by prolonged deoxygenation. However, the extrapolation of the results to the formation of irreversibly sickled cells in vivo has to be made with caution, because additional factors cannot be excluded as contributing to the permanent shape distortion of these cells in vivo.

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REFERENCES

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