Temperature Transitions of Protein Properties in Human Red Blood Cells

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ABSTRACT Human red blood cells (RBC) undergo a sudden change from blocking to passing through 1.3 ± 0.2-μm micropipettes at a transition temperature (T_c) of 36.4°C. For resealed RBC ghosts this transition occurs at 28.3°C (T_g). These findings are attributed to an elastomeric transition of hemoglobin from being gel-like to a fluid and to an elastomeric transition of membrane proteins such as spectrin. Spectrin shows a uniform distribution along the aspirated RBC tongue above T_g in contrast to the linear gradient below T_g.

INTRODUCTION

Cell deformation requires the structural transition and molecular interaction of cellular proteins. When the entropy of a component within a protein system decreases with an increase in temperature, that component is said to have undergone an inverse temperature transition (ITT) (Urry, 1988). Entropic elastomers undergo thermocontraction. This is because of an ITT in which intramolecular hydrophobic interactions are optimized. The development of elastomeric force correlates with the ITT, i.e., with increase in intramolecular order.

Temperature in general, and body temperature in particular, play ubiquitous roles in experimental deformation of cells (Waugh and Evans, 1979; Hochmuth et al., 1980; Nash and Meiselman, 1985a; Sung and Chien, 1992). When inducing extreme deformations of RBCs with a micropipette at room temperature, hidden elasticity and cytoskeletal protein distribution patterns have been observed (Discher et al., 1994; Discher and Mohandas, 1996). These changes, however, were not seen when the same experiments were carried out at body temperature, as almost all RBCs easily passed the 1.3-μm micropipette (Artmann et al., 1997). Considering the above-described elastomeric protein properties, one expects that the elasticity and the in situ cytoskeletal protein distribution patterns to be qualitatively different between room and body temperatures. We show that extreme RBC deformation at a given mechanical load occurs suddenly at distinct critical temperatures. ITTs of cytosolic and cytoskeletal proteins need to be considered. The major intracellular protein in RBCs is hemoglobin. The major RBC membrane protein is spectrin, possessing the properties of thermocontraction (Vertessy and Steck, 1989), reflecting entropy-driven elasticity, and thermoexpansion (Waugh and Evans, 1979), which is favored by the enthalpy component of the elastic free energy of the extended skeleton.

Received for publication 8 May 1998 and in final form 18 August 1998.

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0006-3495/98/12/3179/05 $2.00

We used micropipette aspiration and fluorescence-imaged micropipette tests to study extreme deformations of intact RBCs and resealed ghosts. Two distinctly different critical temperatures for intact RBCs, T_c, and for resealed ghosts, T_g, respectively, were found. This was accompanied by differences in the hemoglobin and spectrin distribution patterns below versus above the critical temperatures.

MATERIALS AND METHODS

To image the lipid bilayer distribution, we used the lipophilic probe DiD (dilC18(5)). This fluorescent label was obtained from Molecular Probes (Leiden, The Netherlands). The DiD-lipid labeling procedure followed that described by Golan et al. (1986).

The micropipette technique was used to test the properties of intact red cells in isotonic media as well as those of resealed ghosts. Glass tubes (10 cm long, 1.2 mm external diameter, 0.68 mm internal diameter, World Precision Instruments, FL), were pulled (Mecanex micropipette puller, Switzerland) to a final internal diameter D_p of 1.1–1.5 μm, resulting in micropipettes of uniform thickness with almost no taper at the tip section. The D_p was determined by light microscopic image analysis using a calibrated scale on a slide as standard. The video observations were carried out using light field, phase-contrast, and fluorescence microscopy. Cells suspended in PBS (137 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 0.7 mM $MgSO_4$, 8.4 mM Hepes, and 4 ml 1 N/l NaOH) were allowed to settle in a glass chamber. A micropipette was placed adjacent to the cell to be tested. A step aspiration pressure was applied. The time taken for the RBC to fully enter the pipette was measured from video recordings for a series of 20–40 cells as the period between the first appearance of a membrane tongue in the pipette and the final disappearance of the portion of the RBC outside the pipette. During each experiment, the same pipette was used to avoid uncertainties associated with measurements of D_p.

The hemoglobin density distribution inside the RBC was visualized by imaging of aspirated RBCs at an illuminating wavelength of 410 ± 6 nm.

For the viscosity measurements, human lyophilized hemoglobin powder (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was diluted in PBS buffer. The relative viscosity η/η_0 of the hemoglobin solutions at various concentrations (where η_0 was the viscosity of PBS and η the viscosity of the PBS with hemoglobin) was measured with a rotational viscometer (Low-Shear 30, Contraves A.G., Zürich, Switzerland) at a constant shear rate of 5.49 s^{-1}. The temperature was raised from room temperature in steps of 0.2° to 50°C.

The method of ghost preparation follows the principles of hypotonic lysis first presented by Dodge et al. (1963). We used the PBS procedure as described by Steck and Kant (1989). The properties of ghosts are critically dependent on the method of preparation (Palek et al., 1971; Nash and Meiselman, 1985b; Scott et al., 1990).

Antispectrin antibodies (Sigma) were conjugated with fluorescein isothiocyanate (FITC, Sigma) (Goding, 1976). This complex penetrated the cell membrane during the lysis procedure followed by resealing, as shown by Dodge et al. (1963).

RESULTS

Transition of red blood cell passage and hemoglobin viscosity at 36.4°C

When we aspirated RBCs at 22°C into 1.3-μm micropipettes, they underwent extreme deformation and the trailing sphere of the RBC blocked the pipette with no possibility of passing (Artmann et al., 1997). Fluorescence intensity mapping of labeled lipids at 22°C (Discher et al., 1994; Bloom and Webb, 1983; Golan et al., 1986) showed that the lipid bilayer uniformly surrounded the cytosol (Fig. 1). As the temperature was raised toward 36°C, a few RBCs became passable. Within a narrow temperature band of 36.4 ± 0.3°C, the RBC passage suddenly changed from ~10% to 90% (Fig. 2, cell passage). The critical temperature (T_c), defined as the temperature at which 50% of RBCs passed the 1.3-μm micropipette, was found to be 36.4°C. At 37°C, a blocking RBC showed a trailing sphere whose diameter decreased slightly during the first 0.1 s after entering the pipette, but remained constant for the ensuing 50 s. The temperature dependency was investigated for a number of passing RBCs. The passage time decreased linearly (r = 0.97) from 10 to 1.7 s as temperature rose from 36.0°C to 38.5°C. RBCs that already had passed the pipette could be easily pushed out of the pipette back into suspension, and they immediately recovered the biconcave discoid shape.

These findings raised the question why RBCs passed above and not below T_c. We investigated the possible roles of intracellular hemoglobin and membrane proteins. Experiments on the same RBC below T_c (35°C, Fig. 3, *A* and *B*) and above T_c (37°C, Fig. 3, *C* and *D*) demonstrated dramatic differences.

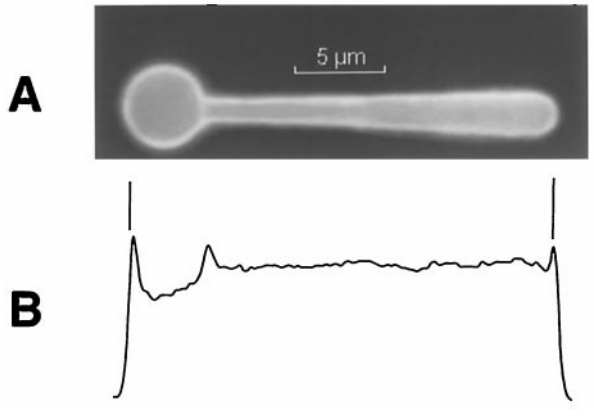

FIGURE 1 (*A*) Fluorescence photomicrograph of an RBC labeled with DiD, in a 1.3-μm pipette aspirated with a pressure of −2.3 kPa (temperature 22°C, isotropic membrane tension T = 6 dyn/cm, the photograph was taken <0.1 s after the RBC entered the pipette). (*B*) Integrated fluorescence intensity for lipid staining as obtained by summation of pixel intensities along the center line.

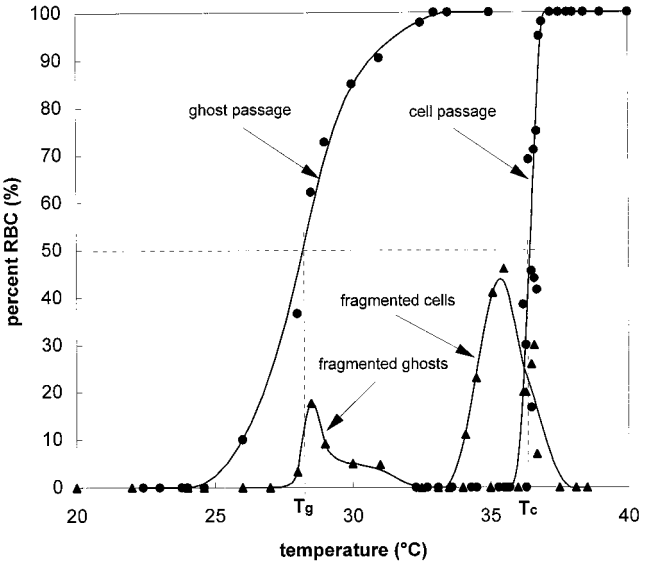

FIGURE 2 *Right curves:* Percent RBC passages through a 1.3-μm micropipette as a function of temperature, showing a critical temperature T_c at 36.3 ± 0.3°C. RBCs passed above and blocked below this temperature range. The percentage of RBC fragmentations observed during pipetting is shown as "fragmented cells," including both passing and blocking RBCs. *Left curves:* The percentage of resealed RBC ghosts passing through a 1.3-μm micropipette (−2.3 kPa), showing a critical temperature at 28.3 ± 2.3°C ("ghost passage"). The percentage of fragmented ghosts, including passing and blocking ghosts, observed during the pipetting is labeled "fragmented ghosts."

By illuminating a blocking RBC at 35°C using monochromatic light at 410 ± 6 nm, optical density changes were seen in the RBC's tongue (Fig. 3 *B*). In addition, we estimated the approximate hemoglobin concentration within the RBC's spherical trail. Assuming no hemoglobin loss occurred during aspiration and only a negligible fraction of hemoglobin was bound to the RBC's tongue and a mean cellular hemoglobin content of $3 \cdot 10^{-11}$ g/cell, an RBC volume of 95 μm^3, we calculated a hemoglobin concentration of ~ 50 g/dl within the spherical trail. This rise in hemoglobin concentration is attributable to the filtration of water across the RBC membrane into the surrounding fluid.

When the same RBC was kept in place and the temperature was elevated above 37°C, the hemoglobin-rich fluid suddenly flushed back from the sphere into the tongue; the hemoglobin concentration rose in the tongue and decreased in the outer sphere. The outer sphere continuously shrank and the RBC eventually passed the pipette. The passing RBC at 37°C showed a relatively uniform distribution of hemoglobin within the tongue (Fig. 3 *D*). The distribution patterns of the optical density (OD) along the pipette axis were distinctly different at 35°C and 37°C. At 35°C, the hemoglobin concentration was elevated immediately after RBC aspiration, although it decreased considerably within

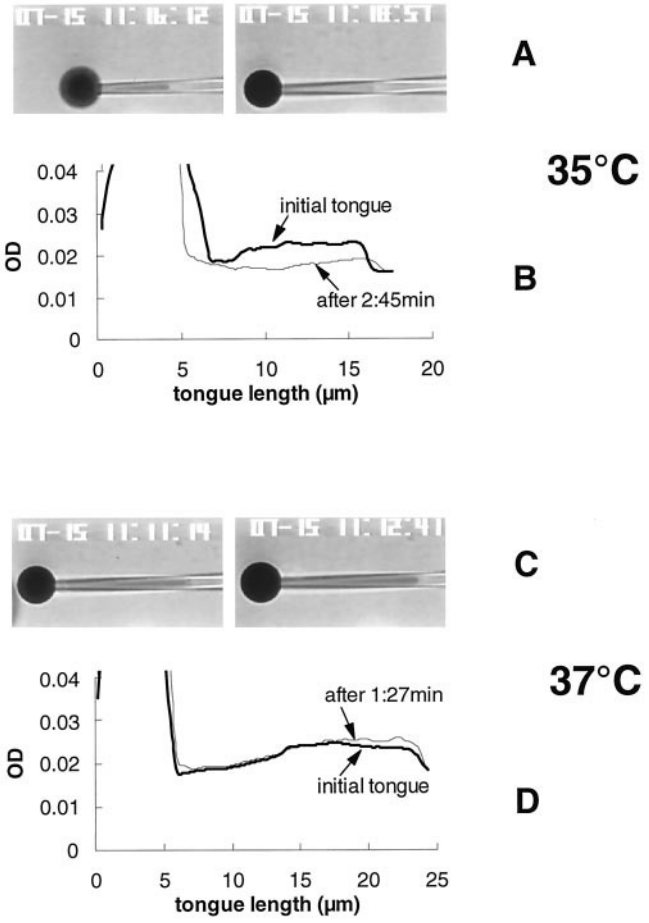

FIGURE 3 (*A*) Videophotomicrographs of an aspirated RBC in a 1.3-μm pipette at 35°C: blocking RBC <0.1 s after entering the pipette (*left*). Hemoglobin was visible in the cell tongue. The same RBC 2 min 45 s later, the hemoglobin in the tongue was clearly depleted (*right*). (*B*) Hemoglobin density distribution at 35°C showing the depletion of hemoglobin in the tongue as a result of pipette aspiration. (*C*) Videophotomicrographs of an aspirated RBC in a 1.3-μm pipette at 37°C: passing RBC <0.1 s after entering the pipette (*left*); the same RBC 1 min 27 s later (*right*). In both photographs, the hemoglobin in the tongue was clearly visible. (*D*) Hemoglobin density distribution at 37°C showing the maintenance of the hemoglobin density in the cell tongue.

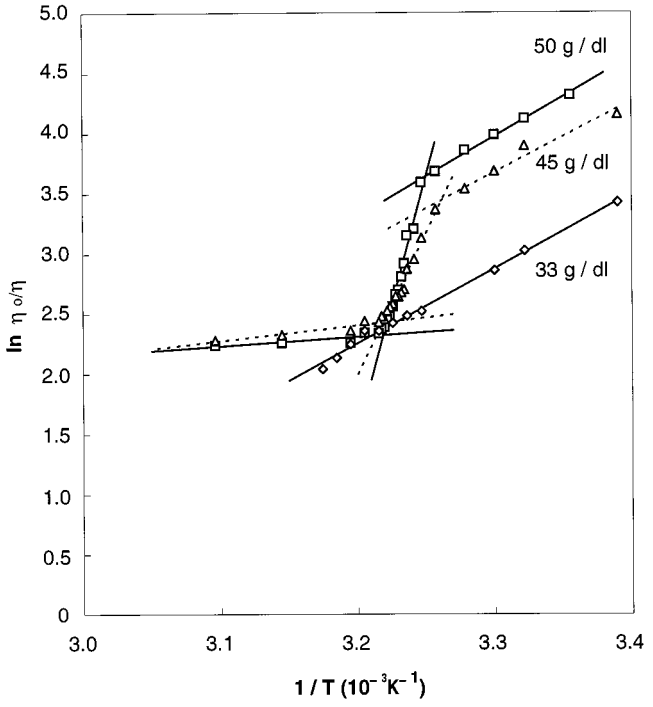

FIGURE 4 Arrhenius plots of the viscosity of human hemoglobin dissolved in PBS at 33, 45, and 50 g/dl. The viscosity-temperature relation was monotonic at 33 g/dl, while at 45 and 50 g/dl it exhibited transitions between 35 and 38°C, with a sudden drop of the viscosity. Each point represents the mean of 10 repeats.

the next 2 min 45 s of recording (Fig. 3 *B*). In contrast, at 37°C the distribution remained almost unchanged (Fig. 3 *D*).

To investigate whether the T_c of 36.4°C could be attributed to a transition of the hemoglobin flow properties, the viscosity of hemoglobin solutions in PBS at different concentrations was measured as a function of temperature (Fig. 4). At a concentration of 50 g/dl, as the temperature was increased, the relative viscosity plotted on a logarithmic scale versus the inverse temperature began to drop sharply at ~37°C. This is qualitatively different from the monotonic, linear relationship between the logarithm of the relative viscosity and the inverse temperature for hemoglobin solutions at the physiological concentration of 33 g/dl (Sung and Chien, 1992). A sharp transition of hemoglobin viscosity at ~37°C was also seen at 45 g/dl, though to a lesser degree than at 50 g/dl.

Transition of red cell ghost passage at 28.3°C and of spectrin distribution pattern

During the micropipette deformation, the cytoskeleton had to undergo extreme extension. To determine whether the cytoskeletal protein network also had a temperature transition, micropipette passage experiments were performed on resealed ghosts (Dodge et al., 1963; Steck and Kant, 1989; Palek et al., 1971; Nash and Meiselman, 1985b; Scott et al., 1990). Although the ghost cells did not show any transition near the body temperature, they exhibited a critical temperature T_g at 28.3 ± 2.3°C (Fig. 2, ghost passage). The passage time of the passing ghosts decreased linearly (r = 0.95) from 28.8 s at 26°C to 16.7 s at 33°C .

Using fluorescence-imaged micropipette aspiration, the density distribution of fluorescently labeled spectrin (Goding, 1976) was determined at 22°C and 27°C (below T_g) as well as above T_g at 29°C and 37°C (Fig. 5, *A* and *B*). The spectrin density of the ghost membrane tongue inside the micropipette decreased continuously along the length at 22°C and at 27°C, but was almost constant at 29°C and 37°C (Fig. 5 *B*). The spectrin density gradient at 22°C is similar to that reported previously (Discher et al., 1994; Discher and Mohandas, 1996). The present study shows that at 27°C there was no qualitative distribution change observed. However, at 29°C as well as at 37°C (above T_g) there was a uniform distribution of the spectrin density along the length of the tongue.

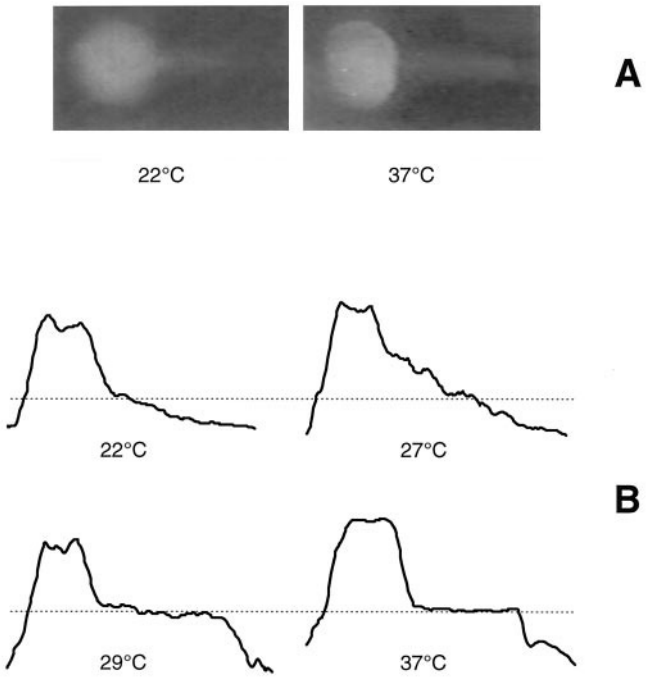

FIGURE 5 (*A*) Fluorescence images of RBC deformation in a micropipette (1.3 ± 0.2 μm, −2.3 kPa) at 22°C and at 37°C. (*B*) Intensity profiles for fluorescein-antispectrin marked spectrin. Note the progressive decrease from the pipette tip to the tongue tip at 22°C and 27°C in contrast to the rather constant intensity profiles at 29°C and 37°C. (The images as well as the fluorescence intensity profiles represent different cells)

Relation of cell fragmentation to transition temperatures

When the micropipette aspiration experiment was performed on intact RBCs, fragmentation (i.e., a part of the RBC's tongue end detached from the rest of the tongue) was observed for both passing and blocking RBCs, with a maximum of 45% of the aspirated RBCs being fragmented at 35°C (Fig. 2, fragmented cells). As the temperature was raised above 35°C, cell fragmentation decreased progressively and disappeared at 37.5°C and above. For resealed ghosts (Fig. 2, fragmented ghosts), the range of temperature transition was much broader than that for intact RBCs, and the maximum ghost fragmentation was 18% at the T_g of 28.3°C. Above the critical temperatures the percentage of fragmentation decreased rapidly with increasing temperature.

DISCUSSION

The fluorescence-imaged micropipette aspiration data on intact RBCs and resealed ghosts, coupled with the hemoglobin absorbance patterns in intact RBCs, demonstrate that protein transitions occur at critical temperatures in RBCs under these conditions.

The passage of intact RBCs through 1.3-μm micropipettes switched from blockage to passage within a narrow range at about body temperature (36.4 ± 0.3°C). In the blocked RBC (Fig. 3, *A* and *B*), the concentration of hemoglobin in the sphere rose to reach 50 g/dl. The estimated water loss of the blocking RBCs was ~20%, which is in agreement with a previous work (Jay, 1973). Viscometric measurements showed that such concentrated hemoglobin solutions had a T_c at essentially the same temperature as RBC passage (Fig. 4). Hence, the high hemoglobin concentration in the sphere formed a highly viscous gel-like cell interior below T_c. At and above the T_c, the hemoglobin solution in the sphere became fluid again and suddenly flew into the tongue as a result of the high membrane tension in the trailing spherical segment. The continued decrease of the sphere diameter finally allowed the cell to pass the pipette. This would require a cytosolic water loss of ~55% after the RBC had completely entered the pipette.

We concluded that the transition of RBCs from blockage to passage at T_c is due to a partial phase separation of hemoglobin and the cytosolic fluid. Inasmuch as the experimental boundary conditions below and above T_c were the same, the hemoglobin must have undergone a transition at T_c in its physical state.

The distribution of spectrin in resealed RBC ghosts as marked by antispectrin antibodies was strikingly different below and above the T_g of 28.3 ± 2.3°C (Fig. 5). In contrast to the considerable axial gradient in spectrin distributions below T_g (Discher et al., 1994; Discher and Mohandas, 1996), the intensity profiles above T_g were rather constant. This, together with the accompanying increase of the percentage of passing ghosts, can be interpreted as a sudden decrease of protein entropy at the transition point when the cytoskeletal proteins were stretched out (Lehninger, 1973). Because the method of ghost preparation we used had a minimal effect on membrane mechanics, the findings on the ghosts are probably applicable to the intact RBC membrane as well (Dodge et al., 1963; Steck and Kant, 1989).

Studies on artificial dimyristoylphosphatidylserine (DMPS)/spectrin vesicles provided evidence that in addition to the binding to band III, spectrin may also couple directly to the lipid moiety of the inner monolayer of erythrocytes through electrostatic coupling to phosphatidylserine (Maksymiw et al., 1987). The authors calculated a transition temperature of 35°C for DMPS/spectrin vesicles. This temperature, however, does not fit the transition we found for resealed ghost at 28.3°C. We conclude that, due to the complex composition of the RBC ghost membranes, the possible phase transition of phosphatidylserine is overwhelmed by the presence of other phospholipids.

The transitions of RBC passage and of RBC proteins are hidden (i.e., cannot be seen) when testing the temperature dependency of material properties of the intact RBCs or ghost membranes by using larger micropipettes (Evans, 1989; Mohandas and Chasis, 1993; Mohandas and Evans, 1994; Chien et al., 1978; Artmann, 1995), because the deformational force applied there is too small in comparison to the entropic elastomeric forces.

The T_c of 36.4°C for the intact RBCs is considerably higher than the T_g of 28.3°C for the ghost membrane, and it may be attributed to the elastomeric fluid/gel-like transition

of hemoglobin. In comparison to the intact RBCs, the T_c for the micropipette passage for resealed ghosts had not only a lower value, but also a broader range. The passing process for resealed ghosts at T_g primarily involved the RBC cytoskeleton, which consists of spectrin and a number of different types of proteins. Each type of cytoskeletal protein probably has distinct elastomeric transitions at somewhat different critical temperatures. In contrast, in intact RBCs the transition was probably attributed mainly to one kind of protein, i.e., hemoglobin, and this resulted in a sharp temperature transition.

Cell fragmentation was related to the transition temperatures in a way that the highest percentage of fragmentations occurred right below T_c and T_g, respectively. This finding suggests that the tension built up below and near the critical temperatures in the cytoskeletal network may cause fragmentation. The transition of proteins at critical temperatures caused a relief of the membrane tension, thus protecting RBCs from being fragmented and allowing them to pass.

In summary, the temperature transitions of micropipette passage and cell fragmentation of intact RBCs and membrane ghosts are accompanied by transitional behaviors of hemoglobin and membrane skeletal proteins, respectively. The passing of an intact RBC through a 1.3 ± 0.2 μm capillary, however, is determined by the hemoglobin physical state, rather than by the cytoskeleton.

These results provide a correlation between rheological behavior at the cellular level and protein properties at the molecular level. It is possible that similar elastomeric transitions of proteins may also occur in other types of cells and affect their functions. Considering RBCs as a prototypical cellular system, we propose that elastomeric temperature transitions may play a significant role in protein dynamics and cell function.

We gratefully acknowledge the critical comments of Prof. Y.C. Fung, University of California -San Diego, Dr. Paola Zigrino, Dept. of Dermatology, University of Cologne, and Dr. Sigrid Zink, Diabetes Research Institute, University of Düsseldorf.

This study was carried out in the Dept. of Cell Biophysics, Aachen University of Applied Sciences. It was supported by a grant of the German Academic Exchange Office, Bonn, to Dipl. Phys. Christina Kelemen, Romania; the German Cellular Engineering work group; and by Dept. of Bioengineering, University of California, San Diego, Grants HL 44147 and HL 43026 to Prof. Shu Chien.

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