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ORIGINAL PAPER

Hemoglobin senses body temperature

G. M. Artmann · Ilya Digel · K. F. Zerlin · Ch. Maggakis-Kelemen · Pt. Linder · D. Porst · P. Kayser · A. M. Stadler · G. Dikta · A. Temiz Artmann

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Abstract When aspirating human red blood cells (RBCs) into 1.3 μm pipettes ($\Delta P = -2.3$ kPa), a transition from blocking the pipette below a critical temperature $T_c = 36.3 \pm 0.3$°C to passing it above the T_c occurred (micropipette passage transition). With a 1.1 μm pipette no passage was seen which enabled RBC volume measurements also above T_c. With increasing temperature RBCs lost volume significantly faster below than above a $T_c = 36.4 \pm 0.7$ (volume transition). Colloid osmotic pressure (COP) measurements of RBCs in autologous plasma (25°C $\leq T \leq$ 39.5°C) showed a T_c at 37.1 ± 0.2°C above which the COP rapidly decreased (COP transition). In NMR T_1-relaxation time measurements, the T_1 of RBCs in autologous plasma changed from a linear ($r = 0.99$) increment below $T_c = 37 \pm 1$°C at a rate of 0.023 s/K into zero slope above T_c (RBC T_1 transition). In conclusion: An amorphous hemoglobin–water gel formed in the spherical trail, the residual partial sphere of the aspirated RBC. At T_c, a sudden fluidization of the gel occurs. All changes mentioned above happen at a distinct T_c close to body temperature. The T_c is moved +0.8°C to higher temperatures when a D_2O buffer is used. We suggest a mechanism similar to a "glass transition" or a "colloidal phase transition". At T_c, the stabilizing Hb bound water molecules reach a threshold number enabling a partial Hb unfolding. Thus, Hb senses body temperature which must be inscribed in the primary structure of hemoglobin and possibly other proteins.

Keywords Red blood cells · Hemoglobin · Temperature transition · Body temperature · Colloid osmotic pressure · Confined water · Glass transition · NMR T_1

Abbreviations

RBC	Red blood cells
NMR	Nuclear magnetic resonance
Hb	Hemoglobin
COP	Colloid-osmotic pressure, temperature sensation

This article is dedicated to Ludwig Artmann who died on July 21, 2001 on a beautiful summer day during which we performed experiments far away. Ludwig Artmann was a man who encouraged us to be strong and to study hard no matter what were the costs.

G. M. Artmann (✉) · I. Digel · Ch. Maggakis-Kelemen · Pt. Linder · D. Porst · P. Kayser · G. Dikta · A. Temiz Artmann
Division Juelich, Aachen University of Applied Sciences, Ginsterweg 1, 52428 Juelich, Germany
e-mail: artmann@fh-aachen.de

I. Digel
e-mail: digel@fh-aachen.de

K. F. Zerlin
Projektträger Juelich, Research Center Juelich, 52428 Juelich, Germany

A. M. Stadler
Institute Laue Langevin, 38042 Grenoble, France

Introduction

Nonlinearities in the physical, biological and chemical properties of cells (Hennig et al. 2002), membranes (Gowrishankar et al. 1998) and biological molecules (Braxenthaler et al. 1997; Pascher 2001) have developed into a growing field of interest. For some types of cells, intrinsic, pronounced nonlinearity is a well known feature, essential for normal cell function and successful signal transduction (Hennig et al. 2002). Some authors even

prefer the term 'phase transition' instead of nonlinearities (Pollack 2001a, b). The concept of nonlinearity and phase transitions in protein and cell function is on the brink of revolutionizing cell biology (Pollack 2001b).

Nonlinear features of hemoglobin (Hb) function have been repeatedly reported (Lukin et al. 2003; Bettati et al. 1998). Allosteric properties regarding its R-T transition and oxygen binding suggest increased dynamics of "ligation intermediate" species and the distinctive role of the hydration shell in Hb function (Knapp et al. 1999; Levantino et al. 2003). For many years it was assumed that the hemoglobin solution viscosity monotonically depended on temperature and concentration (Danish and Harris 1983; Chien et al. 1982; Muller et al. 1992). However, none of these studies used highly elevated, non-physiological Hb concentrations (i.e. significantly higher than 33 g dL^{-1}). In fact, investigators ended their studies at concentrations too low to observe an accelerated viscosity drop around body temperature (Kelemen et al. 2001). In our previous studies on human red blood cells (RBCs) that began a decade ago, sudden temperature-induced changes in passage behavior through narrow micropipettes were found (micropipette passage transition) (Artmann et al. 1998). Hemoglobin was identified as the molecule causing the transition. Further studies on these discontinuities using RBCs and/or standardized Hb solutions showed nonlinear changes at temperatures all close to the human body temperature (Zerlin et al. 2007; Artmann et al. 2004). Lately we showed that Hb obtained from monotreme animals such as the echidna and the platypus, having body temperatures of 33°C, underwent a temperature transition around their respective body temperatures (Digel et al. 2006). All these and further studies (Zerlin et al. 2007) suggested that the structural transition in hemoglobin involving an unknown mechanism is linked to the species 'body temperature'. However, for methodological reasons, many of these studies did not reflect the natural environment which Hb molecules reside in.

This paper aims to show that the sudden structural transition of hemoglobin at body temperature (Artmann et al. 1998) can be shown in a series of other experiments: (1) RBC volume changes as measured with micropipettes, (2) by colloid osmometry, and (3) by NMR T_1 relaxation time measurements (Zefirova et al. 1991) using individual RBCs or whole blood, respectively. The results gave hints on the molecular mechanism of the structural transition. They supported the idea put forward earlier (Kelemen et al. 2001) that a Hb structural rearrangement was initiated by a steady thinning of the Hb bound water shell with rising temperature. Below T_c, the bound water shell stabilized the molecule. At T_c this had reached a threshold. It enabled a molecular partial unfolding (Stadler et al. 2008a) initiating the transitions observed. It occurs in the spherical trail of an aspirated RBC where a Hb–water gel forms below T_c. At T_c this gel suddenly turns fluid caused by a break down of intermolecular Van der Waals forces. The fact that T_c depends on the species' body temperature implies that this temperature is somehow inscribed in the primary Hb structure. Thus, the T_c observed may generally reflect body temperature and the Hb molecule "senses" this particular temperature by undergoing a partial structural transition.

Materials and methods

Blood sample preparation

Blood samples were obtained from healthy donors in EDTA-filled syringes, centrifuged at $500\times g$ for 10 min and with the buffy coat removed from the pellet. Samples were obtained and processed just before the beginning of an experiment. This paper presents data obtained with intact RBCs.

Micropipette measurements

In micropipette experiments used for single cell volume determinations washed RBCs were used. After centrifugation, the RBC pellet was re-suspended in HEPES buffer (pH 7.4, 300 mOsm) at a hematocrit of 0.1%. In a separate set of experiments, D_2O was used instead of H_2O for HEPES buffer preparation.

NMR measurements

In the the NMR as well as in the COP experiments, RBCs were resuspended in autologous blood plasma. The major differences to real blood in the NMR and COP experiments were (1) the absence of shear stress, (2) the absence of leucocytes and thrombocytes, and (3) an elevated hematocrit. The preparation protocols were essential for the results presented below. Plasma and RBC pellets were separated and degassed for 5 min. For samples containing RBC, 0.5 mL plasma and 0.225 mL D_2O were added to 3.5 mL RBC pellet.

Colloid osmotic pressure (COP) measurements

The RBC pellet was re-suspended with autologous plasma. The average hematocrit was $77.6 \pm 5.3\%$ ($N = 16$ blood samples).

Micropipette system set-up

Aspiration experiments for individual RBC volume determination were carried out with an inverted microscope and

bright field light (1,000×) microscopy (CLSM, AxioverT 100, Carl Zeiss, Göttingen, Germany). A micromanipulator system (Luigs and Neumann, Ratingen, Germany), a hydrostatic pressure system and a temperature chamber (Cell & Tissue Technology, Aachen, Germany) at an accuracy of ±0.2°C were used for micropipette experiments. Cylindrical micropipettes with an inner diameter of 1.1 mm were pulled (Mecanex SA, Nyon, Switzerland; BB-CH-PC) from borosilicate glass tubes (World Precision Instruments, Berlin, Germany). The inner diameter was measured microscopically using image analysis. The aspiration pressure was adjusted at −2.3 kPa.

Single RBC volume measurements

For individual RBC volume determinations of $N = 12$ RBCs one and the same micropipette was used. Procedure of volume determination: An RBC was aspirated at $T = 34.5$°C and geometrical data (Fig. 1) were obtained. The cell was then blown out. The temperature was raised by about 0.3–0.4°C and equilibrated. The same cell was re-aspirated and the new geometrical data were measured. The cell was blown out and the temperature was raised again and so on. This way the relative volume change of the particular cell could be measured highly accurate. Cell data could easily be compared with each other since the same pipette was used for all twelve cells. The temperature was adjusted between 34.5 and 39.5°C. In order to avoid irreversible hemoglobin denaturing, 39.5°C was not exceeded (Williamson 1993). For the passage transition experiments in D_2O buffer $N = 40$ RBC were aspirated at each temperature. The same micropipette was used in the measurement series.

RBC volume determinations (Fig. 1a and b) were carried out with established methods (Engstrom et al. 1992). An individual RBC was pulled into the pipette at 34.5°C. The final aspiration pressure $\Delta P = -2.3$ kPa was build up slowly until a steady state was reached i.e. when the RBC tongue length no longer showed any visible changes (Fig. 1a). Thus, microscopic images of aspirated RBCs were acquired at steady state conditions. From these images, geometrical parameters (Fig. 1b) were determined. The RBC volume was calculated as follows (Eqs. 1a–1c):

$$V_1 = \pi/12 \cdot p^3 \text{ (spherical tongue cap)} \quad (1a)$$

$$V_2 = 1/4p^2\pi \cdot (l - 1/2p) \text{ (cylindrical tongue volume)} \quad (1b)$$

$$V_3 = \pi h^2/3 \cdot ((3d/2) - h)), \text{ where}$$
$$h = 1/2d + \left(1/2d^2 - 1/2p^2\right)^{1/2}. \quad (1c)$$

(trail outside the pipette).

The equations describe volumes measured with a circular pipette and cylindrical tip (for abbreviations see Fig. 1b). The total RBC volume was $V = V_1 + V_2 + V_3$.

Hemoglobin molecular radius estimations from micropipette experiments

At an aspiration pressure of −2.3 kPa, the pulling force vector acting on an aspirated RBC tongue and pointing into the micropipette had a higher norm than the colloid osmotic force vector caused by the very high Hb concentration inside the spherical RBC trail (Kelemen et al. 2001) pointing outwards. At this pressure, it was reasonable to assume that most of the cytosolic bulk water had been

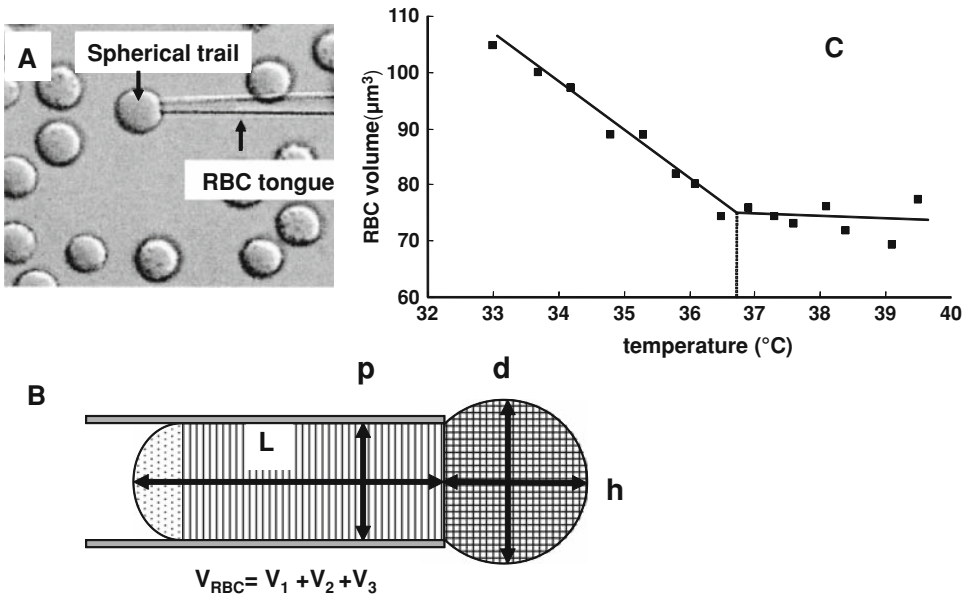

Fig. 1 **a** Typical micropipette aspiration experiment. The *spherical trail* outside the pipette is clearly visible. The *arrow* indicates the end of the aspirated RBC's tongue. **b** Individual volume compartments of an aspirated RBC used for single cell volume calculation. **c** Volume change over temperature of an individual RBC. There was a clear turning point indicating a critical temperature at T_c. The slopes of the *linear curve* below and above T_c differed significantly according to the statistical analysis applied

squeezed out. The cytosolic Hb molecules together with their bound water shell were closely packed, leaving only traces of unbound water within the intermolecular gaps. We have developed a simple geometrical model to estimate the Hb molecular radius, r_{Hb}, under the boundary condition of a circumferential isotropic membrane tension compressing the Hb molecules inside RBCs during aspiration. We considered physiological data as the intracellular Hb concentration in RBCs, $C_{Hb,} = 330$ g/L, molecular weight of tetrameric human Hb, $MW_{Hb} = 64{,}000$ g/Mol, and an average RBC volume of $V_{RBC} = 88$ fL. The molecular Hb radius was calculated using following Eqs. (2a–2c):

$$Hb_{RBC} = c_{Hb} \cdot V_{RBC} \tag{2a}$$

$$N_{Hb} = N_A \cdot Hb_{RBC}/MW_{Hb} \tag{2b}$$

$$r_{Hb} = (3V_{RBC}/4\pi \cdot N_{Hb})^{1/3} \tag{2c}$$

where N_A is Avogadro's number, Hb_{RBC} is the total intracellular Hb content of a single RBC, and where N_{Hb} is the number of Hb molecules per RBC. For the calculation of the number of hydration layers per hemoglobin molecule, it was assumed that the amount of 0.35 g H_2O/g protein corresponds to one hydration layer. A physiological concentration of hemoglobin at 330 g/L in red blood cells at conditions was assumed. The concentration of hemoglobin in the aspirated cells was calculated using the measured cell volume. The amount of cell water was then estimated by assuming a constant density of the hemoglobin solution. The number of hydration layers was finally obtained by dividing the amount of cellular water per hemoglobin molecule by the assumed value for one hydration layer.

NMR measurements

NMR T_1 relaxation time measurements were carried out with samples inserted into NMR tubes at temperatures of 15, 25, 30, 35, 37, 39 and 42°C, respectively. The samples were allowed to equilibrate for 10 min at each temperature step. A DRX 600 (Bruker Co., Germany) NMR device was used for T_1 measurements carried out at a static magnetic field strength of 14.7 T corresponding to a Larmor frequency of 600 MHz (Finnie et al. 1986; Zefirova et al. 1991). An inversion recovery pulse sequence was used and data were analyzed with the XMGR software (http://math.nyu.edu/aml/software/xmgrace.html).

Colloid osmotic pressure measurements

The colloid osmometer (Wescor Co., USA) was placed into a heating chamber at 39°C and the temperature was equilibrated for 30 min. Then the blood plasma-sample was injected into the measurement chamber and the COP was observed. When steady state was reached, this value was taken as the COP of the plasma-sample at this temperature. Afterwards, the sample was exchanged with the RBC-in plasma-sample and the procedure was repeated. Subsequently, the blood plasma and the RBC-in plasma COP were obtained at steps of about 1°C going stepwise down to 29°C.

Statistical data analysis (Dikta et al. 2006)

The volumes of 12 RBCs were considered at temperatures of 34.5, 36.4 (=T_c), and 39.5 (°C). For each cell, three dependent observations were available, while the 12 measurements at each temperature step were independent of each other, since they originated from different cells.

To check whether the expected volume at the temperature $T_c = 36.4$°C was less than the one at 34.5°C, a t-test was applied, where the differences ($D1$) between individual RBC volumes at 34.5°C and $T_c = 36.4$°C were taken as

$$D1 = \text{RBC volume } (T_c = 36.4^\circ\text{C}) - \text{RBC volume } (34.5^\circ\text{C}). \tag{3a}$$

These differences were checked for normality by the Shapiro–Wilks test. If this test showed no significant departure from normality, the one-sided t-test was applicable. The t-test was used to check the null-hypothesis that the expected value of the difference was zero against the alternative that the expected value of the difference was less than zero. A significant departure from the null-hypothesis must therefore be interpreted as a significant negative slope of the first straight line in Fig. 2. The same approach was used to check the second straight line between $T_c = 36.4$ and 39.5°C, i.e. to analyze

$$D2 = \text{RBC volume } (39.5^\circ\text{C}) - \text{RBC volume } (T_c = 36.4^\circ\text{C}) \tag{3b}$$

Since $D1$ and $D2$ can be associated with one of the two slopes, they can be used to check for differences (D) between the two slopes. For each cell, the $D = D2 - D1$ calculation was performed and analyzed statistically as described above.

Results

This paper presents data obtained with intact RBCs from healthy donors. Except for the micropipette experiments in single cell volume determinations, RBCs remained in their natural chemical environment—in blood plasma. There was no shear force acting on the cells during measurements, no leucocytes and thrombocytes were present, and the hematocrit was elevated. These preparation protocols were essential for the results presented below.

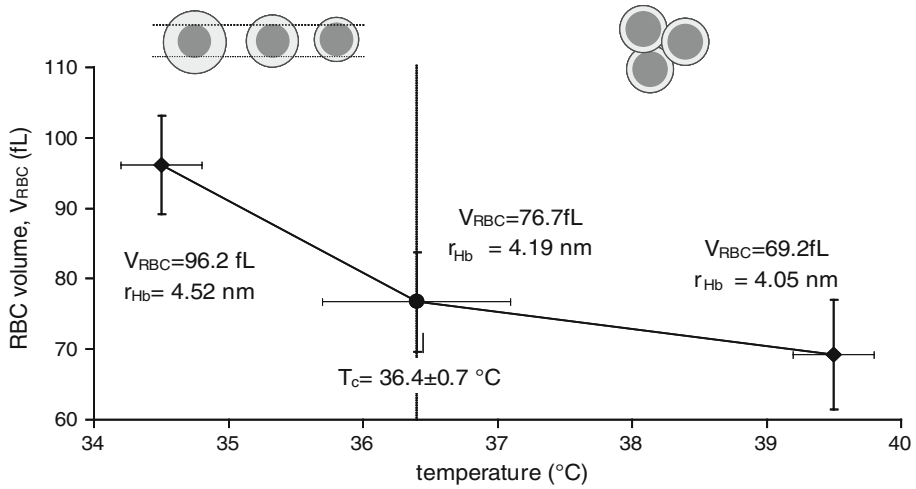

Fig. 2 RBC volume versus temperature during micropipette aspiration (mean ± SD, $N = 12$ cells). The volume of individual RBCs was taken at 15 different temperature steps between 34.5 and 39.5°C at steps of about 0.3–0.4° (compare Fig. 1). For the sake of clarity in this Fig. 2 there were only three data points shown. The RBC volume decreased with increasing temperature but at two significantly different slopes below and above $T_c = 36.4 \pm 0.7$°C, respectively. Below T_c, RBCs lost volume at a rate of 7.8 fL/K, and above at 3 fL/K. The *inserts* represent Hb molecule schemes; *dark:* hemoglobin, *light:* remaining cytosolic water per molecule. On the left: the remaining cytosolic water decreased continuously. Right: The water decreased further, however at a significantly lower rate. The *error bars* in *vertical* direction include systematic and random errors. However, these error bars are dominated by the varying individual RBC volumes

Micropipette experiments

During RBC aspiration (Fig. 1), tongue formation was observed to occur in two time-phases, (1) a fast initial entrance phase which typically lasted less then a second and (2) a subsequent slow creeping phase for a couple of seconds where the tongue length usually changed less then 10%. A steady state of tongue length was considered to be reached, when an aspiration pressure of −12 kPa was applied for a short time and no further tongue length increment was observed. Figure 1c shows a typical volume (Formula 1) versus temperature curve of one individual RBC. The average volume over temperature data of twelve individual RBCs showed a distinct and significant change in slope with a significant kink at $T_c = 36.4 \pm 0.7$ (Fig. 2). The RBC volume below T_c decreased at a rate of 7.8 fL/K with increasing temperature. Setting on at T_c, the rate was decreased to only 3 fL/K. The calculated average RBC volume data at various temperatures are shown in Table 1.

Statistical analysis revealed that the data were normally distributed since the Shapiro test resulted in 0.5029 for $D1$, in 0.6798 for $D2$, and in 0.7433 for D (see Methods). The p-values of the t-test were $p\ (D1) = 0.0000$, $p\ (D2) = 0.0021$, and $p\ (D) = 0.0016$ and confirmed that all three differences deviated significantly from the null-hypothesis. Thus, the slopes between 34.5°C and T_c and between T_c and 39.5°C, respectively were both significantly negative. In addition, the slope in the lower temperature range was significantly more negative than the one above T_c. With Formulae (2a–d) Hb molecular volumes, surfaces and radii were estimated from the micropipette data (Table 1). The Hb radii correspond to a water layer thinning rate per one individual Hb molecule of 0.084 nm/K below T_c and of 0.052 nm/K above, respectively.

NMR T_1 measurements

Two NMR T_1 relaxation time measurements were carried out in parallel (1) with blood plasma containing 83% RBCs (RBC-in-plasma-sample, Fig. 3a) and (2) with blood plasma alone (plasma-only-sample, Fig. 3a). With both samples, a two-phasic behavior of T_1 versus temperature was observed, showing a transition temperature at $T_c = 37 \pm 1$°C. The RBC-in-plasma-sample showed a change from a steady, linear ($r = 0.99$) increment of T_1 below T_c at a rate of 0.023 s/K. Setting on at T_c, T1 did not show any visible dependency of temperature.

The plasma-only-sample showed converse characteristics: No temperature dependency below 37°, a transition temperature at 37°C and a decrease with temperature at the modest rate of 3.8×10^{-3} s/K above T_c (Fig. 3b). The T_1 of the plasma-only-sample was about three to five times higher as compared to the RBC-in-plasma-sample.

Colloid osmotic pressure experiments

As shown in Fig. 4, the COP of the plasma-only-sample increased linearly up to 39.5°C. The COP of the RBC-in-

Table 1 Estimated RBC and hemoglobin molecule data resulting from micropipette experiments

Temperature (°C)	Mean RBC volume (fL)	Hemoglobin molecule volume (nm^3)	Hemoglobin molecule radius (nm)	Number of hydration layers per hemoglobin molecule
25°C	MCV = 88 ± 6	321,6[a]	4.25[a]	6
34.5 ± 1°C	96.2 ± 7	352,0	4.38	7
T_c = 36.4°C	76.7 ± 7.1	280,3	4.06	5
39.5°C	69.2 ± 7.8	252,3	3.92	4

MCV mean cellular RBC volume

[a] Calculated for freely suspended RBCs at an average volume of 88 fL

Please note that Hb molecules were mechanically confined and under elevated pressure due to pipetting

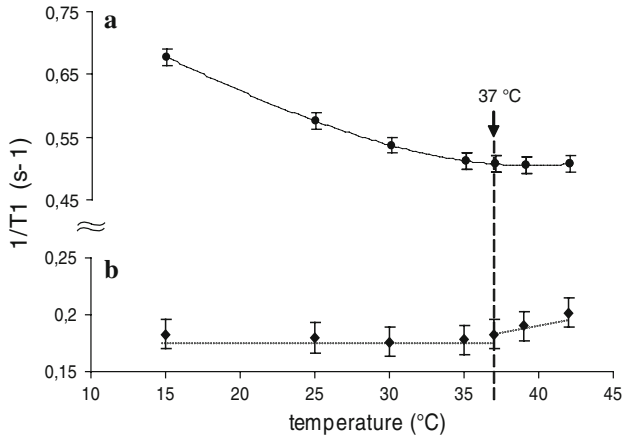

Fig. 3 NMR T_1 relaxation time data. **a** blood plasma containing 83 volume-% RBCs (RBC-in-plasma-sample). The T_1 relaxation time increased with temperature at a rate of about 0.023 s/K below 37°C (310 K). Above 37°C, it remained constant. **b** blood plasma alone (plasma-only-sample). The T_1 change over temperature in the plasma-sample (**b**) showed converse characteristics, i.e. no change below 37° and an increase above 37° (note the different scales $1/T_1$ used in the two graphs)

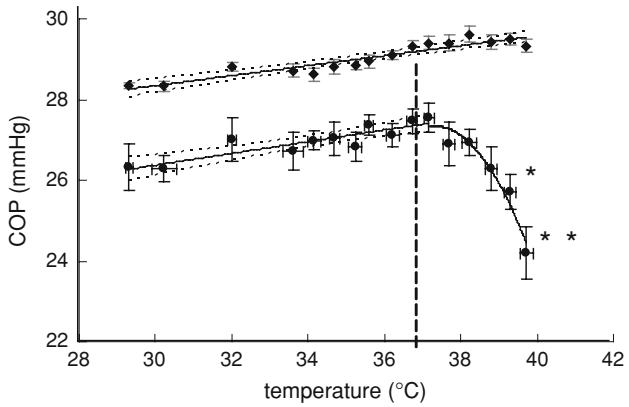

Fig. 4 Temperature course of the colloid osmotic pressure (COP) over temperature. *Upper curve:* Blood plasma without RBC (plasma-only sample), below: RBCs re-suspended in autologous plasma at an average of 77,6 ± 5.3 volume-% RBC (RBC-in-plasma-sample). At each temperature step, $N = 16$ individual samples of healthy donors were measured (average ± 1 SEM). The 95% confidence interval is indicated with *dotted lines*. The RBC-in-plasma-sample showed a 2 mmHg lower COP below T_c and a linear increment with temperature and was in parallel with the plasma-only-sample. Beginning at T_c = 37°C, its COP dropped significantly. Instead, the plasma-only sample showed a regular temperature course with no turning point at 37°C

plasma-sample was in general 2 mmHg lower than the one of pure plasma-samples at the same temperature. Setting on at T_c = 37.1 ± 0.2°C, the COP of the RBC containing sample decreased rapidly and significantly.

Discussion

There is growing evidence that the cell cytosol has the properties of a hydrogel consisting of proteins, protein-bound and bulk water. The current opinion is controversial. Some studies showed that the cell cytosol of cells mainly consists of bulk water (Stadler et al. 2008b; Zaccai 2004), some that the cell content predominantly is gel-like (Pollack 2001a; Pollack 2001b). However, the methods probe either microscopic or macroscopic properties and the results might not be directly comparable. Sudden phase transition like changes of protein function or cell behavior, respectively, could also be related to protein-bound and bulk water effects. In our earlier studies (Fig. 5) a sharp temperature transition of RBC passage behavior through narrow pipettes occurred within less than a degree Celsius at T_c = 36.3 ± 0.3°C (Artmann et al. 1998; Artmann et al. 2004; Kelemen et al. 2001). Moreover, studies with hemoglobins from various species with body temperatures different from those of humans revealed that temperature transitions occurred as well. Those critical temperatures were close to the specific species' body temperature (Digel et al. 2006; Zerlin et al. 2007). We concluded that the hemoglobin molecule senses the species' body temperature by undergoing a partial unfolding at T_c (Stadler et al. 2007; Stadler et al. 2008a). Thus, the critical transition temperature separates two distinctly different physical molecular stages and, consequently, cellular behaviors.

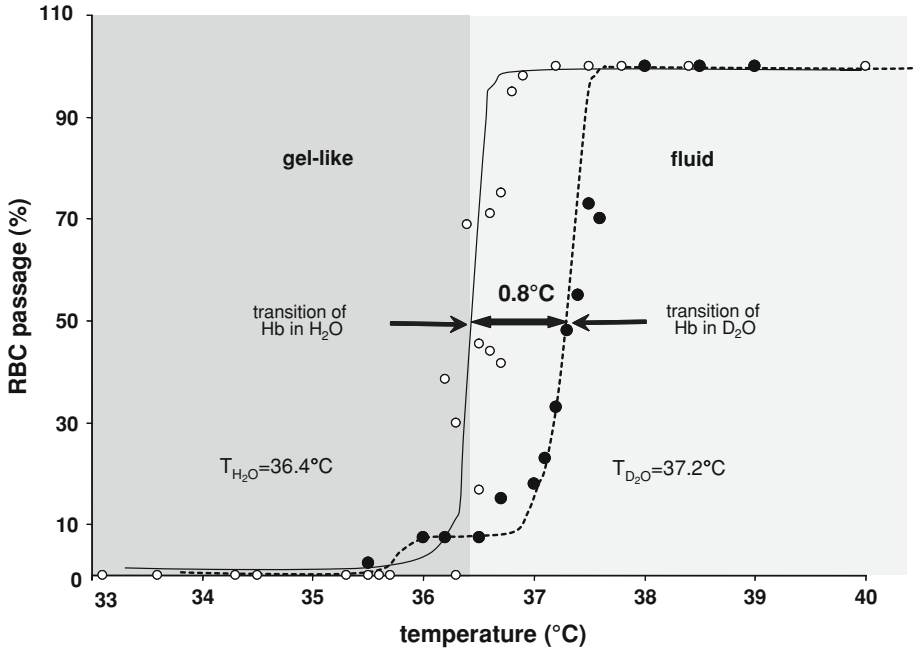

Fig. 5 Micropipette passage experiment. The red blood cell passage curves were obtained with human RBCs. *Left curve* RBCs were suspended in a H_2O based buffer (Artmann et al. 1998), *Right curve* RBCs were suspended in a D_2O based buffer (Buldt et al. 2007; Stadler et al. 2007). The T_c measured with H_2O based buffer was by 0.7–0.8°C smaller then the one with D_2O buffer. This indicated that stronger hydrogen bonds exerted by deuterium replacing hydrogen caused a shift in the critical temperature

Micropipette RBC volume measurements

When an RBC enters a narrow micropipette, an isotropic RBC membrane tension builds up until balance of forces was established (Evans 1983; Hochmuth et al. 1979; Chien et al. 1978). During the aspiration process, cellular bulk water was squeezed out across the membrane and the RBC lost volume (Fairbanks et al. 1971; Farinas et al. 1993; Jay 1996; Poschl et al. 2003; Walz et al. 1997; Yokoyama et al. 1978). In our experiments a short term increase of the aspiration pressure up to −12 kPa did not show any further RBC volume decrement. Thus, the Hb molecules inside RBCs were assumed to be in closest possible contact to each other leaving only traces of bulk water inside the RBC, if at all. The remaining cell water must therefore be trapped between (mostly) hemoglobin molecules and must be constrained in its transversal diffusion (Dencher et al. 2000). This conclusion seems to contradict recent neutron scatter experiments which showed that only 10% of the RBC′s cytosolic water exhibit reduced dynamics (Stadler et al. 2007). However, in the spherical trail of an aspirated RBC the cytosol is compressed due to the aspiration pressure. The remaining distance between adjacent Hb molecules was estimated to be about ten water molecule diameters only (Kelemen et al. 2001). Such protein water arrangement should reduce the translational diffusion of water molecules. A hemoglobin–water-gel had formed in the RBC‘s spherical trail (Fig. 1) which did not allow RBC passage through the pipette below T_c (Artmann et al. 1998).

However, why do at T_c the RBCs set on passing the pipette in an almost step-function like manner? From Fig. 5 must be concluded, that the transition is related to hemoglobin–water interaction. It is known, that hydrogen bonds formed by heavy water are a little more stable than those in normal water. The average protein dynamics was measured with neutron scattering in *Escherichia coli* (Jasnin et al. 2008). Mean protein flexibility and macromolecular resilience (protein ‘stiffness’) were found to be reduced in D_2O compared to H_2O buffer. It was concluded that D_2O favors the packing of non-polar residues in the core of the protein and at the same time enhances the sampling of conformational substates. The reduced protein flexibility and enhanced sampling rate of conformational substates in D_2O would therefore contribute to thermal stabilization of protein structure. Micropipette passage experiments carried out with a heavy water buffer (D_2O buffer) showed a temperature transition at only 0.7–0.8° higher temperature (Fig. 5). Interestingly, in a completely different biological system (*Tetrahymena* cells) the optimum temperature for cell division shifted upward as the heavy water concentration was increased. A maximum shift of 1°C was observed in 40% heavy water (Moner 1972). It is well known from neutron small angle experiments that D_2O causes protein aggregation in certain systems. If only simple protein aggregation would be the cause for the passage phenomenon observed in micropipette experiments, then the usage of heavy water would cause a reduced passage temperature as compared to normal water. However, the micropipette experiments revealed that the passage temperature in D_2O buffer is increased compared to H_2O buffer. Recent neutron scattering experiments with RBCs and hemoglobin solutions, respectively, revealed that at T_c a small partial unfolding of Hb molecules occurred (Stadler et al. 2007; Stadler et al.

2008a; Stadler et al. 2008b). We concluded that this effect initiated the fluidization of aspirated RBCs and their passage through the pipette. Probably the hemoglobin's surface hydrophobicity pattern changed as well which was followed by cytsosolic Hb aggregation. Therefore, the stabilizing effect of heavy water on protein flexibility and on thermal unfolding might be the cause for the increase of the passage temperature observed in micropipette experiments (Buldt et al. 2007; Stadler et al. 2007; Goldenfeld 1992).

RBC volume versus temperature experiments were carried out with micropipettes (Linderkamp and Meiselman 1982). The inner pipette diameter of 1.1 μm was small enough to prevent any RBC passages. Measurements performed between 34.5 and 39.5°C showed that the RBC volume decreased in general with increasing temperature. However, this happened at two significantly different slopes. There was a critical temperature of $T_c = 36.4 \pm 0.7$°C observed where the volume versus temperature curves showed a kink.

The RBC volume of 96.2 fL ± 7.0 at 34.5°C and −2.3 kPa (Table 1) fit to 94.3 fL at −1.96 kPa and to 91.2 fL at 2.45 kPa aspiration pressure at room temperature, respectively, as observed in an earlier publication. With the set of formulae developed for estimating the Hb molecular volume inside an RBC during aspiration (Formulae 2a–d), we determined Hb molecule radii, r_{Hb}, (Table 1). Results on r_{Hb} were surprisingly consistent with those derived in previous light scattering data (Zerlin et al. 2007; Digel et al. 2006). This method, thus, may be a step forward in terms of estimating the Hb molecule radius (Engstrom and Sandstrom 1989; Engstrom and Meiselman 1996).

The major findings of these RBC volume experiments were that RBCs lost volume with increasing temperature significantly more readily below than above T_c. Obviously, the Hb molecules handle water in two distinctly different ways below and above T_c, respectively.

Discussing a decreasing molecular radius for Hb with increasing temperature (Fig. 2; Table 1) seemed to contradict two earlier findings: Light scattering data as well as preliminary X-ray diffraction studies (Buldt et al. 2007) showed increasing hydrodynamic radii and lattice constants with increasing temperatures until T_c was reached. The preliminary X-ray studies with Hb-crystals showed in addition, that the Hb crystal disintegrated at T_c (Fig. 6a).

In postulating two assumptions, a clearer understanding of the volume loss of RBCs with rising temperature was achieved. First, during aspiration the fluctuations of the cytosolic Hb molecules as well as the cell water diffusion

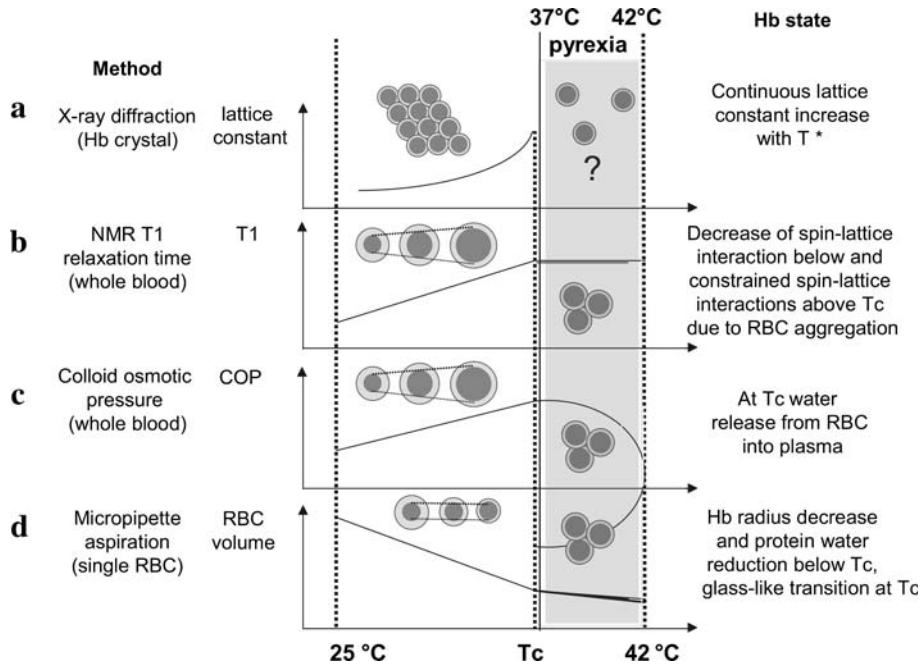

Fig. 6 Summary slide: **a** X-ray diffraction: Below T_c, the Hb crystal lattice constant increases disproportionate with increasing temperature. At T_c, the Hb crystal disintegrates, marked with '?' (Buldt et al. 2007) **b** NMR T_1 relaxation time (RBC in plasma-sample): Below T_c, T_1 increment, at T_c, structural Hb transition, above T_c, no net T_1 change with temperature, **c** Colloid osmotic pressure (RBC-in-plasma-sample): Below T_c, the COP increases, at $T_c = 37.1 \pm 0.2$°C on-set of Hb aggregation causing RBCs to release cell water—COP decreases, **d** Micropipette aspiration (single RBCs): Hb molecule volume remains unchanged with temperature due to hydrostatic pressure inside the spherical trail of the aspirated RBC and bound water turns into bulk water. This was squeezed out due to aspiration pressure. At T_c, a glass-like-transition and further RBC volume loss occur because of hemoglobin aggregation

are mechanically constrained. The Hb molecule could not expand with temperature as it does in free solution and crystals. Water diffusion was as well constricted. Second, the number of water molecules associated with hemoglobin continuously decreased ("melted off") and this water was squeezed out from the cell. This effect caused the RBC volume loss below T_c where the hemoglobin water complex had the consistency of an amorphous gel. The term "melting" used here is just a more descriptive word for a shift of the equilibrium between Hb bound water and bulk water toward bulk water (Cameron et al. 1988).

At T_c the amount of Hb bound water molecules had reached a threshold value at which the Hb–water complex was destabilized. This enabled the partial unfolding and the assumed Hb surface hydrophobicity changes. The latter initiated Hb molecular aggregation which we also found in neutron scattering experiments (Stadler et al. 2007; Digel et al. 2006; Zerlin et al. 2007).

Above T_c, with increasing temperature ongoing cytosolic Hb aggregation determined the RBC volume loss (Fig. 6) due to colloid osmotic effects. We concluded that Hb aggregation lead to an imbalance of the osmotic pressures inside versus outside the cell constituting of native blood plasma. As consequence RBCs release water to the blood plasma to re-balance osmotic pressures. Thus, the COP decreased above T_c with increasing temperature (Fig. 4, see Discussion below).

NMR T_1 measurements

NMR T_1 relaxation time data reflect magnetic interactions of hydrogen nuclei with their environment (lattice). However, interpreting them when the sample consisted of complex protein water solutions is not simple (Victor et al. 2005; Kiihne and Bryant 2000). Although these measurements were not in the focus of this study, they revealed important information. The RBC-in-plasma-sample consisted of 83% RBCs and 17% blood plasma, thus the protons participating in the NMR experiment originated in its majority from water, both protein-bound and free, of hemoglobin, and to a smaller percentage of human albumin. At 83% hematocrit the NMR sample contained 26.4 g dL^{-1} Hb. Thus, the T_1 signal can be attributed to an high extend to Hb–internal proton interactions and Hb–proton interactions with their water–proton environment. We observed a critical temperature at $T_c = 37 \pm 1°C$ (Fig. 3). Below T_c, T_1 rose linearly with increasing temperature. We concluded that these interactions became less and less strong. Since we observed at the same time a "melting-off" of water from hemoglobin (Figs. 1, 2; Table 1) much of this signal increment may have derived from increasing bulk water. At T_c, the T_1 (T)-curve turned into zero slope. Thus, T_c represents the onset of a temperature interval where the T_1 relaxation time became independent of temperature. Interestingly, in the plasma-only sample the T_1 (T)-curve showed exactly a vice versa behavior: Zero slope below T_c and a small negative slope above. In summary, above T_c temperature did not affect any longer the spin lattice interactions in the RBC-in-plasma-sample but began increasing the spin lattice interactions in the plasma-only sample. In other words, in the RBC-in-plasma-sample the intramolecular proton–proton interactions as well as the hemoglobin–proton to water interactions have overruled the interaction of plasma proteins with water. This might arise from the partial unfolding of hemoglobin at T_c and its consequences for hemoglobin aggregation and changes of the hemoglobin bound and free water balance (Artmann et al. 2004; Kiihne and Bryant 2000; Stadler et al. 2007; Stadler et al. 2008a).

Colloid osmotic pressure of RBC suspensions in blood plasma

Some effects in cells can only be seen when cells and proteins are kept in their natural environment (Eisenberg 2003; Zheng and Pollack 2003; Lucas et al. 1991; Todd and Mollitt 1994; Artmann et al. 1998). In our studies, COP data were obtained from both plasma-only-samples and RBC-in-plasma-samples at a hematocrit of 77.6 as function of temperature (Fig. 4). In contrast to micropipette experiments, COP data were gathered both at shear stress-free conditions within the RBC cytosol and at a cell internal hydrostatic pressure unmodified by external mechanical forces acting on RBC membranes (Fig. 6c, d). It was found that (1) the COP of the plasma-only-sample at 29°C fits to the physiological COP found in text books, (2) it increased linearly with temperature, and (3) it did not show any kink (change in slope) at any temperature. However, the RBC-in-plasma-sample as compared to the plasma-only-sample below (1) exhibited a visible but not significant 2 mmHg lower COP, and (2) showed a turning point at $T_c = 37°C$ where the COP began decreasing with temperature (Fig. 4). Thus, a temperature transition was observed in COP measurements at mechanically unaffected conditions.

How can the COP drop above T_c be explained? For low protein concentrations the COP follows van't Hoff's law. In the plasma-only-sample therefore the COP increased linearly with temperature. As for the RBC-in-plasma-sample (Fig. 4), however, things are more complex. Two sample volume compartments must be considered, (1) the volume occupied by RBCs, on average 77.6 ± 5.3%, and (2) the remaining plasma volume at 22.4%. The COP of this sample should be identical to the plasma-only-sample since RBCs are too big to contribute significantly to the COP. In order to understand why the COP of the RBC-in-plasma-samples dropped above T_c, we need to remember

that the COP depends upon the particle number in the RBC cytosol and not on its total protein content. When Hb molecules inside RBCs aggregate as suggested and confirmed earlier (Digel et al. 2006; Zerlin et al. 2007; Stadler et al. 2008a, b) then the particle number and at the same time the intracellular COP decrease. Consequently, cell water moves outwards, diluting the outside plasma until equilibrium is reached. Due to this extra water derived form the RBCs cytosol above T_c the COP of whole RBC-in-plasma-sample decreased.

The global picture

In many experiments on the temperature dependency of Hb properties using light scattering, CD spectroscopy (Zerlin et al. 2007), micropipette volume (Fig. 2) or Hb viscosity measurements (Kelemen et al. 2001) a transition temperature, T_c, was found. However, the transition gradient was never as sharp as in the micropipette passage change (Fig. 5) and in the viscosity experiments of highly concentrated Hb solutions (Kelemen et al. 2001). This implies an unknown mechanism making these two particular transitions significantly sharper. We suggest that T_c marks the temperature at which a colloidal phase transition of second order occurred (Goldenfeld 1992; Heller and Hofer 1975; Landau and Lifshitz 2007). In the micropipette passage experiments, the transition would take place inside the spherical trail of the aspirated RBC where the Hb concentration was between 45 and 50 g dL^{-1} (Kelemen et al. 2001). Below T_c, a "disordered Hb–water gel" was formed consisting of hemoglobin plus bound water and cellular water with dynamics similar to bulk water (i.e. in physical terms "amorphous glass" or "colloidal system with gel-like properties"). Below T_c, sample enthalpy, entropy and volume are continuous functions of temperature. Near the "glass-like-transition" temperature, T_c, the hemoglobin 'glass' or 'colloidal gel' would suddenly "soften" and set on flowing under mechanical cytosolic shear forces exerted when an RBC enters the micropipette (Fig. 5). Above T_c the spherical trail's cytosol (Fig. 2) would exhibit properties approaching those of an ordinary fluid (Artmann et al. 1998; Kelemen et al. 2001), although still more viscous than low-molecular weight liquids. We suggest that the very sharp micropipette passage transition represents a phenomenon similar to a "glass transition" or "colloidal phase transition" common to many polymers and proteins (Craig and Terentjev 2005; Hill et al. 2005; Smith et al. 2004). In strict classical sense the term "glass transition" or "dynamic transition" is used for a change in protein dynamics at 200 K (−73°C). However, some "glass transition" features fit surprisingly well to the properties of the temperature transition observed in the micropipette experiments.

What would now be the links of physics to physiology? Our answer is that T_c 1) marks the set-point of a species body temperature originating from a partial unfolding of hemoglobin at T_c (Digel et al. 2006; Zerlin et al. 2007; Stadler et al. 2008a; Stadler et al. 2008b), 2) T_c represents the set point of a reversible Hb denaturation (Artmann et al. 2004), finally, T_c marks the beginning of the pyrexia zone on the temperature scale. In this pyrexia zone, depending on the actual stage of cell internal Hb aggregation followed by COP changes cell water moves in and out of the cell depending on the direction of temperature change (Fig. 4). The latter might contribute to blood homeostasis during fever. The pyrexia zone in humans ends physiologically at a temperature, $T_{ID} = 42{,}6°C$, where proteins thermally denature irreversibly. The pyrexia zone might now be defined by two distinct temperatures, the lower one marked by T_c and the upper one marked by T_{ID}. Evidently, new perspectives on cell biophysics using completely different approaches from those used by molecular and cell biologists will become more and more important in our attempt to understand cells as gels, and muscles as engines based on principles of polymer science.

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