

HEAT DENATURATION OF ISOLATED ERYTHROCYTE SKELETONS. THERMAL DIELECTROSCOPY DATA

I. Ivanov*, B. Paarvanova

Department of Physics, Biophysics, Rentgenology and Radiology; Medical Faculty, Trakia University, Stara Zagora, Bulgaria

ABSTRACT

Under-membrane cytoskeleton of enucleated erythrocytes represents a lattice of peripheral proteins, mainly spectrin and actin, responsible for the unique mechanical properties of erythrocytes. We used thermal dielectroscopy to study the denaturation temperatures of human erythrocyte cytoskeletons after their extraction from isolated erythrocyte ghost membranes with Triton-X-100. Heating suspensions of these Triton-X-shells two irreversible sigmoid changes in suspension capacity and resistance were registered indicating thermal denaturations of proteins. The first change represented an increase, and the second one a decrease, in suspension capacitance and resistance, without dispersion between 50 kHz and 5 MHz. The mid-temperatures of these changes were very close to the denaturation temperatures of spectrin and of band 3 protein, respectively. In contrast to the first denaturation temperature, the second one demonstrated high sensitivity to the presence of Triton-X-100. The latter temperature was irreversibly increased by appr. 10 °C after covalent binding to Triton-X-shells of 4,4'-diiso-thiocyanato stilbene-2,2'-disulfonic acid (DIDS), specific inhibitor and powerful thermal stabilizer of band 3 protein. We assumed that the indicated changes in the electric properties of Triton-X-shells involved the heat denaturations of spectrin and band 3 protein demonstrating that the thermal dielectroscopy could give additional information about the under-membrane skeleton of erythrocytes.

Key words: anion exchanger, red blood cells; impedance spectroscopy, spectrin skeleton, thermal methods.

INTRODUCTION

The most abundant cells in human body are erythrocytes the main function of which is to supply oxygen to tissues and to remove carbon dioxide of them. To carry out their mission the erythrocytes are endowed with a plasma membrane, comprising of two layers; lipid bilayer with intercalated integral proteins and a highly elastic protein network, juxtaposed beneath the former. The unique mechanical properties of erythrocytes membrane is due to the indicated under-membrane network of peripheral proteins, mainly spectrin and actin, known as under-membrane erythrocyte skeleton, or erythrocyte skeleton, for short (1). The RBC deformability and elasticity are important contributors to the viscosity of blood

and their impairment has been correlated with many cardiovascular diseases, such as myocardial infarction, diabetes mellitus, essential hypertension, hereditary spherocytosis, sickle cell anemia, and malaria (2, 3, 4, 5, 6). In addition to erythrocytes, similar submembrane spectrin-actin network of filamentous proteins has been identified in a wide variety of nonerythroid cells in mammals and birds (7).

Enucleated erythrocytes itself and their hypotonically isolated plasma membranes are frequently used in model studies of cells and plasma membranes in general. In addition, erythrocytes provide yet another useful model for membrane investigations. When the isolated plasma membranes of erythrocytes are subjected to solubilization by a mild, polar detergent such as Triton-X-100, bare erythrocyte under-membrane skeletons, frequently referred to as Triton X-shells, are produced. The latter represent delipidated

*Correspondence to: Ivan Tanev Ivanov: Dept. Physics, Biophysics, Rentgenology and Radiology, Medical Faculty, Thracian University, Armeyska Str. 11, Stara Zagora 6000, Bulgaria. E-mail: ivanov_it@gbg.bg

under-membrane protein network devoid of most of the membrane integral proteins. The shells consist exclusively of spectrin and actin, and a small amount of band 3 protein, an integral protein known as anion exchanger (AE1, the chloride/bicarbonate exchanger) (8). The main virtue of Triton X-shells is that they maintain the same two-dimensional organization of the erythrocyte spectrin-actin under-membrane lattice and, consequently, the shape of parent erythrocytes. Hence, the main use of Triton X-shells has been in the investigations of the structure and composition of erythrocyte membrane skeleton and its molecular defects that underlie several types of hemolytic anemias.

Thermal denaturations of major proteins appear as separate peaks on the differential scanning calorimetry thermogram of isolated erythrocyte membranes. The denaturation of spectrin occurs at 49.5 °C (9), the cytoplasmic domain of the band 3 protein at 62 °C, and the membrane domain of the band 3 protein at 67 °C (10). Little, if any, data exist for the thermal denaturation of isolated Triton X-shell skeletons. The aim of this study was, using thermal dielectroscopy method (11,12), to detect thermal denaturations of isolated Triton X-shell skeletons. The obtained data could be used in future studies to gain additional information about the main protein components of erythrocyte membrane.

MATERIALS AND METHODS

Red blood cells were isolated from freshly drawn human blood and thrice washed in excess cold 150 mM NaCl saline. The erythrocytes were immediately lysed in a 10 volumes of a cold (1 °C) medium, containing 4 mM MgCl₂ and 5 mM Tris-HCl buffer, pH 7.8. 5 min after the lysis the isotonicity was restored to 150 mM NaCl adding a quarter volume of cold 0.75 M NaCl. The erythrocyte ghost membranes obtained were isolated (8000 rpm, 12 min) and immediately subjected to solubilization mixing the membranes with a quarter volume of NaCl saline that contained 4 mM MgCl₂ and 1% (w/v) Triton-X-100. The solubilization of membranes lasted 2 hours at cold (4 °C). Finally, the obtained solution was mixed with 3 volumes of cold NaCl saline, containing 4 mM MgCl₂ and centrifuged (8000 rpm, 12 min) to sediment and isolate the Triton X-shells (erythrocyte skeletons).

The Triton X-shells were isolated as a white pellet and immediately studied or, prior to study, they were additionally washed one or three times in excess volume of NaCl saline, containing 4 mM MgCl₂. These skeletons are further referred to as one-step and three-step washed skeletons, respectively.

The extraction of erythrocyte ghosts with the nonionic detergent Triton X-100 solubilizes all of the integral membrane proteins and most of the lipids; but spectrin, actin, and a minor group of other cytoplasmic membrane proteins remain insoluble. According to (8) the Triton X-100 insoluble cytoskeleton (shell) fraction of human erythrocytes consists of spectrin, actin, bands 4.1 and 4.2, and a fraction of band 3 protein (10-15% of its original content).

In this study we used thermal analysis of the admittance of suspensions that contained Triton X-shells (erythrocyte membrane skeletons). The method was explained in details earlier (11, 12) and only a brief overview is provided below. First, 70 µl of the isolated erythrocyte membrane skeletons were placed in a conductometric cuvette that contained two platinum wire electrodes distanced at about 3 mm from each other, Φ 4 mm. The cuvette was put into a hole drilled into an aluminum block and the block was heated with constant heating rate of 3.0 °C /min within the indicated temperature interval. During the heating the suspension admittance was measured at the indicated set of frequencies with Solartron 1260A Impedance/Gain-phase analyzer, England, connected to computer. The electric properties of a given suspension under study were expressed by an equivalent electric scheme containing a resistance, R, in parallel with a capacitance, C. From the collected admittance data the values of R and C were calculated for each frequency and temperature. Finally, the temperature profiles of the resistance, R, and capacitance, C, were plotted at each frequency chosen.

RESULTS

Figure 1 (A and B) displays the temperature profiles (thermograms) of the resistance, R, and capacitance, C, of a suspension, containing one-step washed isolated erythrocyte skeletons. Shown are the temperature-induced changes in R and C, relative to the respective values of R₀ and C₀ at the initial temperature (appr. 20 °C). Each curve was obtained at the

indicated frequency. Clearly distinguished are two processes on the thermograms of suspension resistance and capacitance. These processes are outlined by sigmoid changes that overlie upon the pure, Boltzmann type of temperature dependence of suspension R and C. The mid-temperatures (46 °C and 55 °C, respectively) of these processes are indicated by arrows. The first one corresponds to a huge

sigmoid increase in capacitance, C, and similar, although much smaller, increase in the resistance, R, of the suspension. Conversely, the second process indicates a huge sigmoid decrease in capacitance and similar, although much smaller, decrease in the resistance of the suspension. These processes could reflect the heat denaturation of proteins, associated with the isolated erythrocyte skeletons.

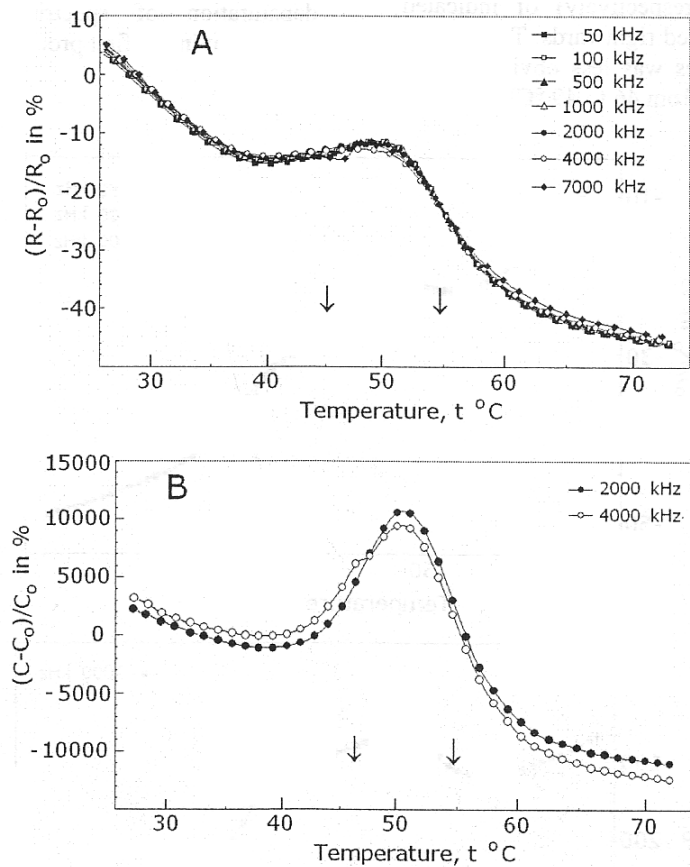


Figure 1. Temperature dependence of the resistance, R, (A) and capacitance, C, (B) of suspension of one-step washed erythrocyte under-membrane skeletons. The latter were isolated through solubilization of erythrocyte ghost membranes with 0.2 % (v/v) Triton-X-100. The suspension medium contained 4 mM MgCl₂, 150 mM NaCl and about 0.002% (v/v) residual Triton-X-100. Protein content and the heating rate were about 3 mg/ml and 3 °C/min, correspondingly. Frequency of the measuring current is shown in the insert. Arrows indicate the mid-temperatures of two sigmoid – type changes on the thermograms of R and C.

The coincidence of all curves obtained at different frequencies (**Figure 1**) indicates absence of dielectric relaxation processes, including the important beta-dispersion. The latter is due to the Maxwell-Vagner effect of interfacial accumulation of free charges on the both sides of closed membranes. This type of interfacial polarization is obligatory demonstrated with whole erythrocytes and resealed erythrocyte ghost membranes within

the 0.1-10 MHz frequency range and with liposomes in the 10 - 300 MHz frequency range (13). The absence of beta-dispersion with the suspension of Triton-X-shells could be due to the Triton-X-shells being spherical lattice or network of cross-linked proteins. The one-step washed isolated erythrocyte skeletons contained residual amounts of Triton-X-100 that could reduce the denaturation temperatures of its proteins.

Consequently, we studied Triton X-shells subjected to more rigorous washing procedure in order to fully remove the detergent and eliminate its labilization effect on the proteins of Triton X-shells.

The temperature curves of R and C of three-step washed erythrocyte skeletons are displayed in **Figure 2A** and **Figure 2B**, respectively. In this case, the mid-temperatures (49°C and 65°C, respectively) of indicated processes were shifted rightwards. The shift in the mid-temperatures was less obvious at the first process (3 °C, from 46 to 49 °C) and more

pronounced at the second process (10 °C, from 55 to 65 °C). This indicates the first process had much smaller sensitivity to the labilization effect of Triton X-100 in respect to that of the second one. In addition, according to its mid-temperature and half-peak width the first process fully coincided with the heat denaturation of the major protein of erythrocyte membrane skeletons, spectrin. Based on these findings, we assume the heat denaturation of spectrin was the main participant in the first process on thermograms.

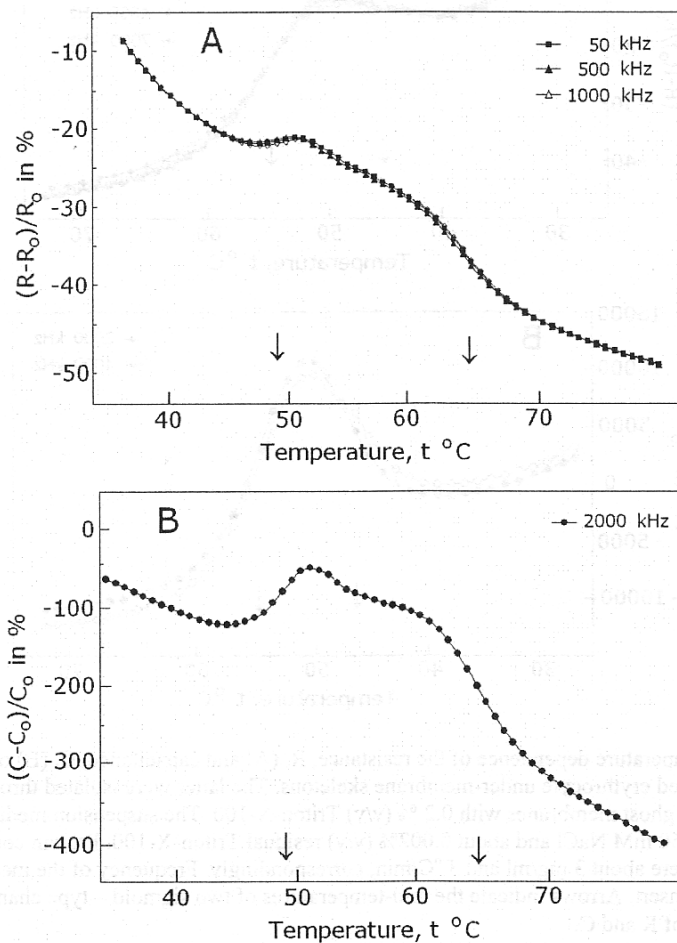


Figure 2. Temperature dependence of the resistance, R, (A) and capacitance, C, (B) of suspension of three-step washed erythrocyte under-membrane skeletons. Other details were as indicated for the **Figure 1**, except the suspension medium did not contained Triton-X-100 due to the multi-step washing procedure.

With three-step washed isolated erythrocyte skeletons the second process was significantly distanced and well separated from the first one (**Figure 2**). It apparently corresponded to the heat denaturation of a different, significantly

more stable membrane protein than spectrin. To disclose the nature of this protein we added DIDS (4,4'-diisothiocyanato stilbene-2,2'-disulfonic acid), to a final concentration of 50 μ M, during the first washing of the Triton X-

shells. DIDS is a bifunctional, covalent amino reagent, specifically binding and cross-linking the polypeptides of the anion exchanger protein of erythrocyte membranes (10). At low concentrations ($< 50 \mu\text{M}$) DIDS binds specifically to the anion exchanger increasing its denaturation temperature by about $13 \text{ }^\circ\text{C}$, from $67 \text{ }^\circ\text{C}$ to $80 \text{ }^\circ\text{C}$ (14). In our case (**Figure 3**) the covalent binding of DIDS to the three-step washed Triton X-shells shifted the mid-temperature of the second process from $65 \text{ }^\circ\text{C}$ to $75 \text{ }^\circ\text{C}$.

There are plethora of reports on the structure of the band 3 protein and its interaction with DIDS (15). With a molecular weight of about 95 kDa the band 3 polypeptide forms two domains. The 40 kDa N-terminal domain is located within the cytoplasm of erythrocytes and serves as a binding site for the undermembrane skeleton, while the 55 kDa C-

terminal domain is membrane-inserted. On the other hand band 3 protein exists as a mixture of dimers and larger oligomers, the predominant (70%) species being a dimer (16). It could not be dissociated to monomers, other than by protein denaturation. The fraction of band 3 not associated with the cytoskeleton is almost entirely dimeric. The higher oligomers interact with the cytoskeleton, and are held together by interactions of the cytoplasmic domain. There is one DIDS binding site per each band 3 polypeptide thus one molecule of DIDS cross-links two band 3 polypeptides.

Based on above considerations and the result, demonstrated in **Figure 3**, we assume that the second process on thermograms (**Figures 2, 3**) could involve the heat denaturation of the transmembrane domain of the anion exchanger.

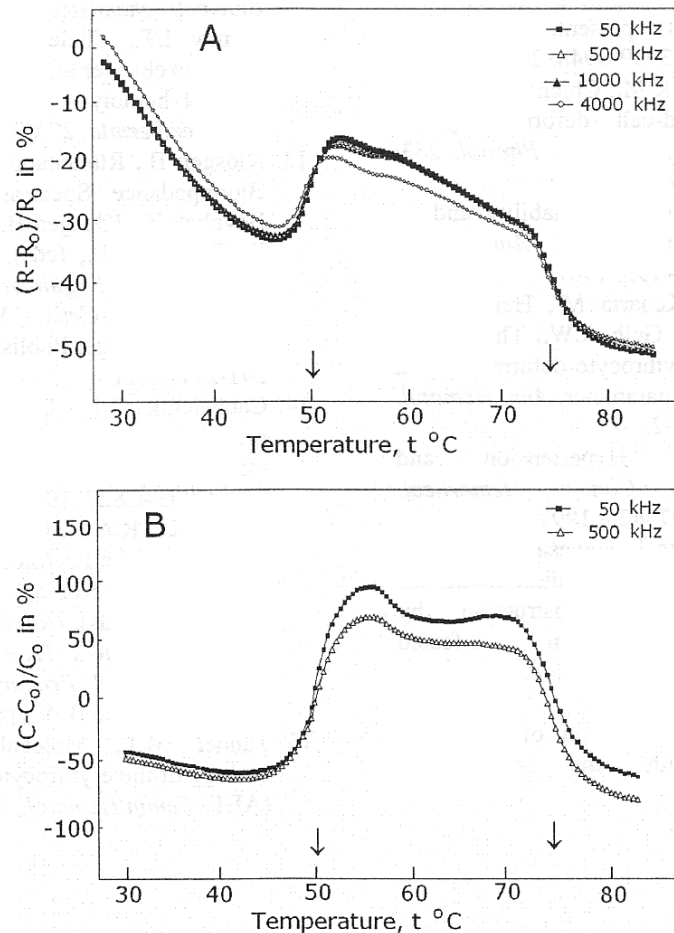


Figure 3. Effect of DIDS on the temperature dependence of the resistance, R , (A) and capacitance, C , (B) of suspension of three-step washed erythrocyte under-membrane skeletons. During the second washing the isolated skeletons were treated with $50 \mu\text{M}$ DIDS. Other details were as indicated for the **Figure 2**.

In conclusion, thermal dielectroscopy data of isolated erythrocyte membrane skeletons allow the registration of two irreversible thermal denaturations as sigmoid changes in the thermal profiles of their passive electric properties. The low temperature (50 °C) change could be associated with denaturation of spectrin while the high temperature (65 °C) one could be ascribed to the heat denaturation of the band 3 protein. During both denaturations the suspension capacitance and resistance changed in the same direction; the change in capacitance being more than 10 folds greater compared to the change in resistance. Hence, the changes in suspension resistance appear consecutive to the changes in suspension capacitance which indicates a transition in the dipole moments and dielectric polarizability of the heated undermembrane skeletons.

REFERENCES

1. Mohandas N., Gallagher P. G., Red cell membrane: past, present, and future. *Blood*, 112 (10): 3939-3948, 2008.
2. Simchon S., Jan K.M., Chien S., Influence of reduced red-cell deformability on regional bloodflow. *Am. J. Physiol.* 253: H898-H903, 1987.
3. Chien S., Red-cell deformability and its relevance to blood-flow, *Annu. Rev. Physiol.* 49: 177-192, 1987.
4. Mokken F.C., Kedaria M., Henny C.P., Hardeman M.R., Gelb A.W., The clinical importance of erythrocyte deformability, a hemorheological parameter, *Ann. Hematol.* 64: 113-122, 1992.
5. Ajmani R.S., Hypertension and hemorheology, *Clin. Hemorheol. Microcirc.* 17: 397-420, 1997.
6. Shelby J.P., White J., Ganesan K., Rathod P.K., Chiu D.T., A microfluidic model for single-cell capillary obstruction by *Plasmodium falciparum* infected erythrocytes. *Proc. Natl. Acad. Sci. U.S.A.* 100: 14618-14622, 2003.
7. Mangeat P.H., Interaction of biological membranes with the cytoskeletal framework of living cells, *Biol. Cell.* 64: 261-281, 1988.
8. Sharma S. and Gokhale S.M., Solubility Behaviour of Integral Proteins And Glycophorins of Mammalian Erythrocyte Membrane. *Asian J. Exp. Biol. Sci.* 2(3): 449-454, 2011.
9. Brandts J.F., Erickson L., Lysko K., Schwartz A.T., Taverna R.D., Calorimetric studies of the structural transitions of the human erythrocyte membrane. The involvement of spectrin in the A transition. *Biochemistry*, 16: 3450-3454, 1977.
10. Snow J.W., Brandts J.F., Low P.S., The effects of anion transport inhibitors on the structural transitions in erythrocyte membranes. *Biochim Biophys Acta*, 512: 579-591, 1978.
11. Ivanov I.T., Impedance spectroscopy of human erythrocyte membrane: Effect of frequency at the spectrin denaturation transition temperature. *Bioelectrochemistry*, 78:181-185, 2010.
12. Ivanov I.T., Zheleva A., Zlatanov I., Anion exchanger and the resistance against thermal hemolysis. *International Journal of Hyperthermia*, 27 (3):286-296, 2011.
13. Klösgen B., Rügenapp C. and Gleich B., Bioimpedance Spectroscopy. In: Boöf-Bavnbek B., Klösgen B., Larsen J., Pociot F., Renström E., (eds), *BetaSys: Systems Biology of Regulated Exocytosis in Pancreatic β -Cells*. Vol. 2 Systems Biology. Springer Publishing Company, pp 241-271, 2011.
14. Cabantchik Z.I., Greger R., Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol*, 262: C803-C827, 1992.
15. Reithmeier R.A.F., Chan S.L., Popov M., Structure of the Erythrocyte Band 3 Anion Exchanger. In: Konings WN, Kaback HR, Lolkema JS (eds), *Handbook of Biological Physics. Volume 2. Transport Processes in Eukaryotic and Prokaryotic Organisms*. Elsevier Science B. V., pp 281-309, 1996.
16. Tanner M.J., Molecular and cellular biology of the erythrocyte anion exchanger (AE1). *Semin Hematol*, 30: 34-57, 1993.